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THE SPECIFIC NATURE OF THE INHIBITION OF THE COAGULATING EFFECT EXERTED BY TISSUE EXTRACT ON PLASMA RESULTING FROM INCUBATION OF TISSUE EXTRACT WITH BLOOD SERUM[1]

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It has been known for many years that substances, which cause or accelerate the coagulation of the blood, occur not only in the blood serum (thrombin and its precursor substances) but can also be extracted from various tissues, such as kidney, liver, muscle and from the cells circulating in the blood, in particular from the erythrocytes (1a). These substances which are contained in the tissues have received different names, such as thrombo-plastic substances (A. Schmidt, Howell), tissue fibrinogen (Wooldridge, Mills), cytozym (Fuld and Spiro, Bordet) and thrombokinase (Morawitz, 9). These terms in part at least represent different theories concerning the mechanism by means of which these substances convert the fibrinogen of the blood plasma into fibrin; thus the term "tissue fibrinogen" indicates that fibrin consists of a combination of blood fibrinogen and tissue fibrinogen, while the term "thrombokinase" signifies that this substance acts as an activator which converts the thrombogen into prothrombin. We used in our earlier investigations the term tissuecoagulin for this substance, which did not imply a theory as to the mechanism underlying its action. In 1903 and 1904 Loeb (1, 1a) and Hewlett (2) and soon afterwards Muraschew (3) and Nolf (4) showed that a specific relation exists between the tissuecoagulins and the kind of plasma on which they act. The tissuecoagulin derived from a certain class of animals caused coagulation relatively more efficiently when it acted on the blood of an animal belonging to the same class than on that of another class. This

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specific relation between tissue coagulin and blood plasma we designated as a specific adaptation between these two substances and the substances as such were specifically adapted to each other. Subsequently Loeb and Fleisher (5) showed that a further going specific adaptation can be demonstrated in the interaction between the coagulins extractable from mammalian erythrocytes and mammalian blood plasmas. In this case a specific adaptation can be demonstrated not only in different classes of animals, but also in different species and orders. Thus it is possible by means of this specific adaptation to distinguish the erythrocytes of rabbit, cat and dog. Experiments, in which these specifically adapted coagulins, blood serum and plasma were quantitatively varied, indicated that in addition to the activating effect, which in accordance with the findings of Morawitz these substances exerted on certain constituents of the blood plasma, they caused or accelerated the coagulation of the blood also by a direct action on the fibrinogen of the blood plasma. This conclusion received confirmation by studies of the coagulation of invertebrate blood, in which it could be shown that the tissuecoagulins convert the fibrinogen directly into fibrin (1a). These substances were diminished in activity by heating to 56° or 60°; they were therefore not entirely heat stable.

Such a specific adaptation between tissuecoagulin and plasma suggested that proteins are involved in the action of the tissuecoagulins. However subsequently Bordet and Delange (6), Howell (7) and Zak (8) showed that certain lipoid substances, which Howell identified with kephalins, are the agents present in various tissues which accelerate coagulation. More recently Kraus and Fuchs (14), as well as Fischer (15), concluded that tissue extracts contain two active agents, namely, 1, a lipoid substance, cytozym, and 2, a substance identical with the active substance present in the blood, namely, prothrombin. The latter is assumed to be responsible for the heat lability of the tissue extracts. These heat labile substances, when changed into thrombin, do not act as a kinase, but convert the fibrinogen directly into fibrin. As stated above, addition of tissue extract to blood serum may cause a considerable increase in the activity of the latter; this effect has been attributed to the action of the lipoid cytozym on a precursor substance of thrombin. In the presence of Ca this activated precursor substance becomes converted into thromin.

However it was found, on the other hand, that addition of blood serum to tissue extract under certain conditions may lead to a weakening of the coagulating effect of the tissuecoagulins and that this weakening effect increases with increasing incubation of this mixture (Loeb, 10). In many cases the tissuecoagulin thus is made entirely inactive and such a mixture may be wholly deprived of its coagulating effect. There were moreover indications that a specific adaptation, similar to that between tissuecoagulin and blood plasma, exists also in the case of the inhibiting effect

which develops when tissue extract, serum and plasma, interact with each other (10). The species or class relationship between the animals, from which serum, tissue extract and blood plasma were derived, seemed to influence in a specific manner the intensity of this inhibiting or neutralizing effect. In addition to this indication of a specific adaptation, there was found a difference in the absolute quantity of inhibiting substance present in various sera, the latter being especially great in dog serum. There were furthermore indications that mammalian erythrocytes, which exhibited a marked specific adaptation between their tissue coagulins and the blood plasma on which they acted, were very poor in the inhibiting substance which presumably combines with a constituent of the blood serum (11).

There exist, therefore, as far as their effect on the coagulation of blood is concerned, two different kinds of interaction between blood serum and tissue extracts, one leading to an acceleration of the coagulation of blood plasma, the other one leading to an inhibition of this process, both these processes depending upon the length of time during which serum and tissue extract interacted previous to the addition of their mixture to the plasma; but in these two cases the time curve was different. In general, the accelerating effect predominates if the mixture acts on plasma which is prevented from spontaneous coagulation by inactivation of calcium through NaFl or other substances having similar effects to NaFl; in this case it is mainly the serum fraction (thrombin) of the mixture which induces coagulation. On the other hand, the inhibiting effect predominates, when the mixture is allowed to act on hirudin or peptone plasma; in this case it is the tissuecoagulin which is the active ingredient of the mixture, which causes coagulation. Phosphorus poisoning in dogs destroyed the inhibiting component of the blood serum; also heat injured the inhibiting substance (Loeb, Fleisher and Tuttle, 11).

The specific relations between sera and tissue extracts, as far as the inhibiting effect of these combinations is concerned, was investigated further by Burns, Scharles and Aitken (12). They found that especially with homologous combinations, when sera and kidney extracts were derived from the same species, the inhibiting effects predominated on incubation of these mixtures, whereas the inhibition on standing was much less marked, when heterologous combinations were used, in which sera and organ extracts belonged to different species and classes of animals. An exception to this rule occurred when ox and sheep serum were combined with extracts of these two species. In this case evidently the production of substances accelerating the coagulation of blood was so marked in homologous combinations, that it overbalanced the inhibiting or neutralizing effects; thus the inhibiting effects were slight throughout, but they were somewhat greater in combination with heterologous than with

homologous kidney extracts. The opposite condition prevailed in certain respects with dog serum; this contains a very large quantity of inhibiting substance and in the combinations of dog serum the inhibiting or neutralizing effects were especially pronounced in homologous combinations with extracts.

The interpretation of the results of these experiments is evidently complicated by the fact that, if we combine blood sera and organ extracts from various species or classes of animals, substances intensifying as well as preventing the coagulating effect of sera and extracts develop in the combinations and that in certain combinations the one effect predominates, while in others the other predominates. It seemed to us of great interest to establish still more definitely these specific relationships (specific adaptations) between sera and organ extracts for two reasons. In the first place the existence of such a specific adaptation between two substances very strongly points to the conclusion that proteins are involved in these reactions. We assumed therefore at first that tissuecoagulins are proteins and when it was later found that lipoid substances were able to activate the precursor substances of thrombin present in blood serum and that such activating substances were present in tissue extracts, we believed it probable that these agents accelerating the coagulation of the blood represent combinations of proteins and lipoids. A similar view was expressed by Mills (12). A corresponding conclusion might apply also to the substances responsible for the development of the inhibiting or neutralizing processes which can be observed in the coagulation of the blood if blood serum acts on tissue extract. The establishment of these specific relationships or adaptations between sera and extracts should therefore aid in the analysis of the nature of the substances which interfere with and control the coagulation of the blood.

But there is a second reason which induced us to study further these relationships between sera and organ extracts derived from different species or classes. The fact that a specific adaptation exists between such substances seems to us a fact of general biological significance. It indicates the presence of conditions normally which can be produced experimentally by immunization.

For these reasons we decided to analyze still further the factors on which the mode of interaction between sera and organ extracts from different species or classes of animals depends,—an interaction which may lead either to an acceleration or to an inhibition of the coagulation of the blood. In order to obtain conditions as uncomplicated as possible, we restricted ourselves in these new experiments to the study of various combinations between avian serum and avian extract, on the one hand, and of dog serum and dog extract on the other hand. We tested the interaction of these substances in homologous as well as in heterologous combinations. In

carrying out these experiments, we considered the time during which serum and extract were incubated; we also varied the concentration of the tissue extracts and furthermore we tested the effect of heating the various extracts at 56° or 60° for 30 minutes preceding the preparation of the mixtures. Lastly we investigated also the effects of hemolysed avian and dog blood corpuscles alone, as well as in combination with sera, on the coagulation of the blood. These latter experiments are however of only restricted value because, in accordance with our previous observations, we noted that goose erythrocytes exert a relatively very slight coagulating effect on blood plasma.

METHOD. The procedures used in these experiments were very similar to those used by Burns, Scharles and Aitken. The amount of serum in every case was 0.7 cc., of extract 0.3 cc. and of plasma 1 cc. The periods of incubation of the mixtures of blood sera and extracts were, as a rule, 2, 10, 20, 40 and 80 minutes. At the end of each period, the blood plasma was added and the time required for coagulation noted. In control experiments, 0.7 cc. of a 0.85 per cent NaCl solution was substituted for blood serum. Both dog plasma and goose plasma were used; the former was prepared by adding heparin in the proportion of 1 mgm. per 6 cc. of blood, while goose plasma was obtained by centrifugation of goose blood which had been collected in paraffined tubes without addition of heparin.

Two kinds of kidney extracts were employed, a concentrated and a diluted one. In preparing the former, the kidney was first washed with 0.85 per cent NaCl solution, cut into small pieces and mashed in a mortar; 0.85 per cent NaCl solution was then added in the proportion of 1 gram of mashed kidney to 1 cc. of NaCl solution. This mixture was kept in the icebox over night and on the following morning was filtered through a Buchner funnel with filter paper. As a rule 1 cc. of dog kidney extract thus prepared caused coagulation of dog plasma in about 30 to 40 seconds. In order to obtain the diluted kidney extract, the concentrated extract was diluted with 0.85 per cent NaCl solution so much that coagulation occurred in four to five minutes. Dog serum was prepared from blood obtained from the jugular vein (by means of a cannula); this was placed in the refrigerator over night, when the serum had separated from the clot. Similarly goose blood was collected in large centrifuge tubes from the jugular vein, a small piece of blood clot or of a vein having previously been placed at the bottom of the tube. After standing over night in the refrigerator, the blood was centrifuged and the serum pipetted off. The stroma of dog erythrocytes was obtained from defibrinated dog blood; after separation from the serum by centrifuging, the red blood corpuscles were washed four or five times with 0.85 per cent NaCl solution. The corpuscles from 100 cc. of blood were then suspended in 50 cc. of 0.85 per cent NaCl solution and twice the amount of distilled water was added.

After standing, the stromata were separated from the hemoglobin by centrifuging. The goose blood corpuscles were centrifuged directly and the plasma pipetted off; they were then treated in the same way as the dog corpuscles.

We shall give only a résumé of our various experiments. Each combination of serum, plasma and heated and unheated, concentrated and diluted kidney extract was tested in a number of experiments, which varied in different cases; however the results of the individual experiments were sufficiently in agreement with each other to justify the construction of average curves of the clotting of the plasma, under the influence of the different combinations of serum and kidney extract or of serum and a suspension of stroma of erythrocytes. We shall reproduce the average curves obtained with the various combinations.

In a first series of experiments, we tested the effect of dog serum and goose serum, and of dog and avian kidney extract, as well as of the suspensions of erythrocytes, uncombined, on dog and goose plasma. We had thus an opportunity to test again the degree of specific adaptation of these substances to the coagulable substance in mammalian and avian blood plasma. This series was then followed by experiments in which the various combinations of serum and extract or suspension of erythrocytes stromata were tested.

1. *Effect of dog serum and goose serum on the coagulation of dog plasma and goose plasma.* *a.* Dog serum with goose plasma: Coagulation usually took place in less than 10 minutes; 0.5, 1 and 2 cc. serum were about equally effective. *b.* Goose serum with goose plasma: Coagulation was very much less rapid than with dog serum. In one half of the experiments, coagulation took place after about 12 hours; in the other half, 15 minutes to $2\frac{1}{2}$ hours were required. In a few experiments, no coagulation took place. The larger quantities of serum were, on the whole, more effective than the smaller ones. *c.* Goose serum with dog plasma: Dog plasma coagulated much more slowly than goose plasma. In the majority of cases it occurred after 12 hours; in some experiments no coagulation took place. The larger quantities of serum were more active than the smaller ones. *d.* Dog serum with dog plasma: with $\frac{1}{2}$ cc. serum, coagulation did not take place or it occurred only after many hours' standing; with 2 cc. serum, coagulation occurred in one-half the cases within a few minutes or a few hours, but in one half of the cases even here 12 hours were required. With intermediate quantities of serum the coagulation times were intermediate.

From these experiments we may conclude that 1, dog serum causes more rapid coagulation of both goose plasma and dog plasma than does goose serum; 2, goose plasma undergoes coagulation more rapidly than dog plasma with goose serum as well as with dog serum; and 3, no specific

adaptation is therefore noticeable between sera and plasmas of different classes of animals.

2. *Effect of homologous and heterologous kidney extracts on dog plasma and goose plasma.* Homologous combinations in which extract and plasma were obtained from the same species or class of animals were much more effective than heterologous combinations, in which extract and plasma were derived from different species or classes; this difference was especially marked when smaller quantities (0.1 and 0.2 cc.) of extract were used. In one experiment 0.1 cc. dog kidney extract caused coagulation of dog plasma in 10 minutes while 0.2 cc. was effective in 1 to 2 minutes. With goose plasma which as stated above clotted with serum more rapidly than did dog plasma, no coagulation took place with 0.1 cc. dog kidney extract; with 0.25 cc. dog kidney extract, clotting occurred only after many hours over night. Conversely chicken kidney extract caused the coagulation of dog plasma much more slowly than that of goose plasma.

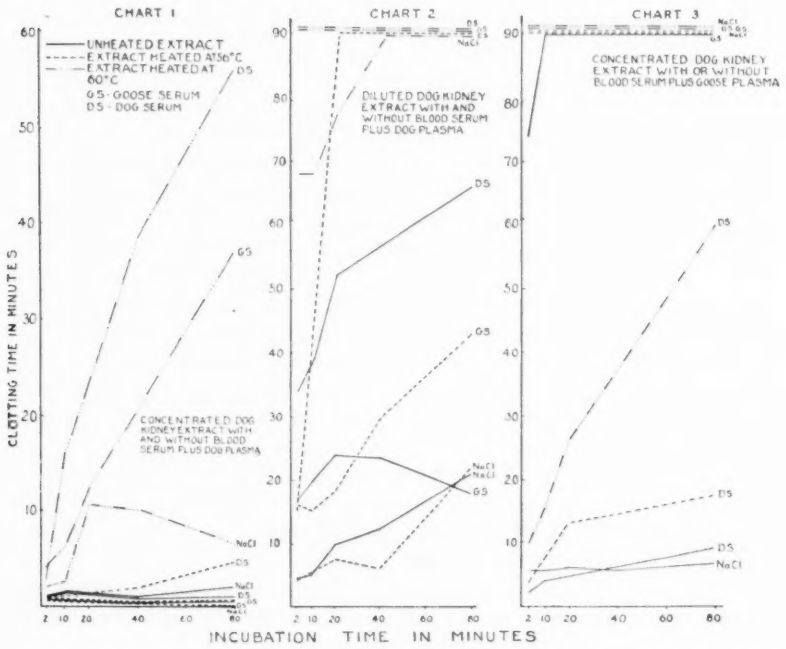
3. *Effect of suspensions of stroma of blood corpuscles.* These were less effective than kidney extracts; but in this case also specific adaptation was evident. Stroma of goose blood corpuscles caused the coagulation of goose plasma more rapidly than of dog plasma. Stroma of dog blood corpuscles induced more active clotting of dog plasma than of goose plasma. As much as $\frac{1}{2}$ cc. and 1 cc. of these suspensions were used, because very small quantities were not effective. We may thus conclude that a specific adaptation exists between tissue extracts or suspensions of erythrocytes and plasmas obtained from the same species or class of animals. These observations were extended in the experiments which follow and the results obtained agree with the conclusions here outlined and with those of previous years.

We next tested the influence which incubation with serum exerts on the ability of the various extracts to induce coagulation of dog or goose plasma. In each case after completion of the incubation time, the plasma was added to the mixture of serum and extract.

4. *Action of dog kidney extract, with or without dog or goose serum, on the coagulation of dog plasma.* a. *Action of concentrated dog kidney extract.* (Chart 1.)[2] In a mixture of 0.3 cc. concentrated dog kidney extract with either 0.7 cc. 0.85 per cent NaCl, dog serum or goose serum a coagulation

[2] In constructing the curves representing the clotting times of the plasma under the influence of the extracts alone and of the extracts in combination with sera as functions of the incubation time of the mixtures of sera and extracts, we considered only the first 90 minutes after addition of tissue extracts and sera to plasma, although we had extended our observation to a period of about 20 hours. As far as these curves are concerned, plasmas which had not yet clotted after 90 minutes were considered as non-coagulable. The lines signifying the different degrees of heat to which the extracts were exposed, previous to their addition to sera, are the same as those used in the other charts.

of 1 cc. dog plasma occurred in a few minutes; this held good even after a long period of incubation of the mixture of dog kidney extract and serum previous to the addition of dog plasma. Heating of dog kidney extract at 56° for 30 minutes did not noticeably weaken the extract, when 0.3 cc. of the latter was combined with 0.7 cc. NaCl solution; but heating it at 60° for 30 minutes caused a slight weakening. If dog kidney extract heated at 56° was first incubated for 80 minutes with dog serum the coagulation of the plasma was retarded for a few minutes, whereas with goose serum no inhibition was noticeable. After incubation of dog kidney extract heated at 60° with dog serum, inhibition of coagulation occurred,

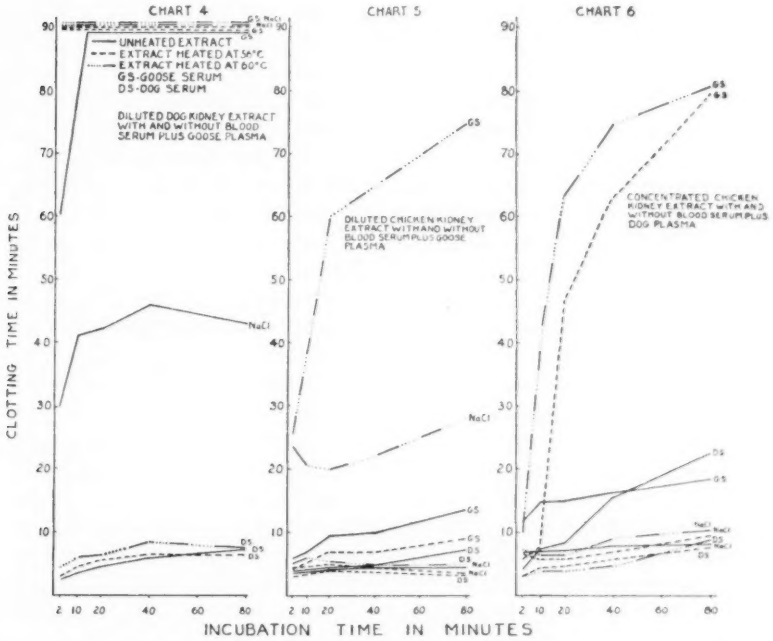


which was greater the longer the period of incubation. If the same kind of extract was incubated with goose serum instead of with dog serum the inhibition of the coagulation of the plasma was much less marked or absent.

b. *Action of diluted dog kidney extract.* (Chart 2.) In combination with 0.85 per cent NaCl solution, the coagulation was much slower than with concentrated extract. Dog serum in combination with dog kidney extract caused an inhibition which increased with increasing incubation, while in combination with goose serum there was no or almost no inhibi-

tion of coagulation. Diluted dog kidney extract, heated at 56° for 30 minutes, alone, acted like unheated diluted dog kidney extract, but incubation of this heated extract with dog serum caused a marked inhibition of the coagulating effect of the extract, which increased with increasing length of incubation. After incubation of the heated extract with goose serum the inhibition was much less. Diluted dog kidney extract heated at 60° for 30 minutes was injured to such a degree that it caused coagulation neither in combination with dog serum nor with goose serum.

From these experiments we may conclude that dog serum acting on dog plasma inhibits in a specific manner the coagulating action of dog kid-



ney extract, as compared with goose serum which has a very much less inhibiting effect. However in order to make this inhibiting action, which increases with increasing incubation, manifest, it is necessary to weaken concentrated dog kidney extract either by diluting it or by heating it, previous to its mixture with serum and the subsequent addition of the incubated mixture to plasma. If the extract used is too concentrated, the inhibiting action of serum does not become sufficiently evident; on the other hand, if the weakening of the extract through dilution or heating has been too great coagulation does not take place at all. Heating at

56° weakens the extract very slightly or not at all; but heating at 60° causes a more definite weakening of the extract. This effect of heating or dilution on the extract may become evident only if the extract is combined with the inhibiting serum.

5. *Action of dog kidney extract, with or without dog or goose serum, on the coagulation of goose plasma.* a. *Action of concentrated dog kidney extract.* (Chart 3.) Three-tenths cubic centimeter dog kidney extract with 0.7 cc. of 0.85 per cent NaCl solution caused coagulation of goose plasma, but less actively than of dog plasma. After heating dog kidney extract at 56° or at 60° it no longer caused clotting of goose plasma. If the unheated dog kidney extract was combined with dog serum, the effect was similar to that in the control experiment, in which the extract was combined with 0.85 per cent NaCl solution; but when goose serum was substituted for dog serum, there was a marked inhibition of coagulation, the coagulation time increasing sharply with increasing time of incubation of serum and extract. As stated, with dog kidney extract heated at 56° alone, clotting did not take place; addition of goose serum did not make the heated dog kidney extract active, but a mixture of dog serum and heated dog kidney extract caused clotting fairly rapidly, namely, between about 3 and 17 minutes, the rapidity of coagulation decreasing with increasing length of incubation of dog serum and dog kidney extract.

With dog kidney extract heated at 60° the results were similar in every respect to those obtained with the 56° extract, except that a combination of dog kidney extract heated at 60° with dog serum was somewhat less active and that the effectiveness decreased still more with increasing incubation than was the case with dog kidney extract heated at 56°.

b. *Action of diluted dog kidney extract.* (Chart 4.) Three-tenths cubic centimeter diluted dog kidney extract plus 0.7 cc. 0.85 per cent NaCl solution caused a moderately active coagulation of goose plasma, on the average in about 15 minutes; but it was less effective than diluted chicken kidney extract. If diluted dog kidney extract was combined with dog serum the coagulation of goose plasma was accelerated, while in combination with goose serum again a marked inhibition occurred as compared with the control in which 0.85 per cent NaCl took the place of serum. This inhibition increased rapidly with increasing length of incubation of diluted dog kidney extract with goose serum. With dog kidney extract, heated at 56°, plus NaCl, no clotting of goose plasma took place; similarly after addition of goose serum to the diluted kidney extract no coagulation of goose plasma occurred, but such dog kidney extract in combination with dog serum caused rapid coagulation.

We see then that in principle the same effects on the coagulation of goose plasma were noted with the various combinations of dog kidney extract and sera irrespective of whether extracts were concentrated or diluted.

With goose plasma, dog kidney extract loses its coagulating power by heating it at 56° for 30 minutes. Dog serum increases the coagulating effect of dog kidney extract with goose plasma, whereas goose serum diminishes it. However in order to bring out these effects clearly dog kidney extract must be neither too strong nor too weak. If it is too concentrated, the accelerating effect of dog serum may be covered up; if, on the other hand, it is too weak, the inhibiting effect of goose serum may be covered up, because in this case the extract as such is unable to cause coagulation. Dilution and heating of dog kidney extract act in principle in the same manner, both causing a weakening of the extract; but, in contrast to what we found with dog plasma, heating at 56° destroys the ability of dog kidney extract to cause clotting of goose plasma because dog kidney extract is less efficient with goose plasma than with dog plasma to which latter it is specifically adapted.

The specific adaptation in regard to the inhibiting action in this case does not relate to the extract, but to the plasma used; as in all the other cases it is the homologous combination (goose serum with goose plasma), which inhibits, while with the heterologous combination of dog serum with goose plasma an accelerating effect preponderates. Although heated extracts of dog kidney, if acting on goose plasma are inactive, they become effective in association with dog serum.

6. *Action of chicken kidney extracts, with and without sera, on the coagulation of goose plasma.* Heating of concentrated and diluted chicken kidney extract at 56° for 30 minutes does not noticeably weaken its clotting action on goose plasma (chart 5); but heating at 60° does somewhat weaken it. Combination of extract with dog serum causes no inhibition; it may even, if favorable conditions are given, produce some acceleration, while after incubation with goose serum the inhibition is more marked. The specific inhibition may in this case depend either on the relation between serum and extract or on that between serum and plasma or on both. In heterologous combinations between serum and extract, an acceleration of the coagulation of goose plasma may occur; such an effect occurred when chicken kidney extract was incubated with dog serum.

7. *Action of chicken kidney extract, with or without sera, on the coagulation of dog plasma.* Heating concentrated chicken kidney extract at 56° and 60° does not seem to cause a marked weakening of the extract, if extract alone acts on dog plasma (chart 6); still some change has taken place in the extract as the result of the heating. This becomes manifest, when we combine extract with serum. After even a relatively short incubation with goose serum, there is a very marked inhibition, while after incubation with dog serum there is indication of an acceleration of the coagulation of dog plasma. In this case the inhibition cannot be due to a specific adaptation between blood serum and plasma, but it must be due to a spe-

cific adaptation which exists between serum and chicken kidney extract. The homologous combination causes inhibition, while the heterologous combination tends to cause acceleration. The corresponding experiments with diluted chicken kidney extract could not be carried out, because the latter was too weak to cause coagulation of dog plasma.

8. *Action of the stroma of dog and goose blood corpuscles on dog plasma and on goose plasma.* While the action of the stroma of dog blood corpuscles and goose blood corpuscles on dog plasma and goose plasma is very specific, each kind of corpuscles being specifically adapted to its own plasma, no definite specificity is recognizable, when dog serum or goose serum is added, and an increase in inhibition under the influence of serum is lacking in the majority of cases. The inhibiting factor, which combines with serum, is evidently not present to the same extent in blood corpuscles as in kidney extract. Heating of blood corpuscles at 56° and 60° did not as a rule weaken the coagulating power of the blood corpuscle-stroma to the same extent as it did that of kidney extract, although heating of dog blood corpuscles at 60° diminished somewhat the coagulating effect of the stroma of such erythrocytes on dog plasma.

Discussion. Our results can be summarized as follows: Dog kidney extract and chicken kidney extract act in a specifically adapted manner on dog plasma and goose plasma. In the case of dog serum and goose serum such a specific adaptation to these plasmas is not demonstrable. As might be expected, dilution and heating of the extract act in a similar manner, both diminishing the quantity of the coagulating substance present in a unit volume of fluid and thus giving the various kinds of serum a chance to exert more efficiently their specific inhibiting effect on the coagulating action which extract alone would have exerted on blood plasma. Also the accelerating effects which combinations of serum and extract exert under certain conditions may come out more clearly with diluted than with concentrated extract in some cases. It can thus be shown that dog serum tends to accelerate the coagulation of goose plasma, which takes place under the influence of dilute dog kidney extract and chicken kidney extract, and that goose serum inhibits the coagulation of goose plasma otherwise exerted by these extracts. On the other hand, it can also be shown that dog serum inhibits dilute dog kidney extract when it acts on dog plasma, whereas goose serum does not exert an inhibiting effect under these circumstances. Dog serum accelerates the coagulating effect of concentrated chicken kidney extract on dog plasma, whereas goose serum inhibits it. Likewise, dog serum does not inhibit the effect of concentrated dog kidney extract on goose plasma, while goose serum inhibits it. On the other hand dog serum but not goose serum inhibits the action of dilute dog kidney extract on dog plasma. Conversely, dog serum does not inhibit but it may even accelerate the effect of chicken kidney extract on goose plasma, whereas goose serum does inhibit this effect.

In general, the results obtained with these different combinations are consistent. The effects as a rule come out most clearly in a middle zone of dilution, where the concentration of the extract is not so strong that addition of serum becomes ineffective and not so weak that it has become unable to cause coagulation; in the latter case an inhibiting effect of serum would no longer be demonstrable.

Our findings here are essentially in agreement with our former observations and also with the observations of Burns, Scharles and Aitken, which indicated that a specific relation exists between the nature of the extract and of the blood serum. Dog serum inhibits the effect of dog kidney extract on dog plasma, while it accelerates the effect of chicken kidney extract on dog plasma. Goose serum inhibits strongly the effect of chicken kidney extract on dog plasma, but it does not inhibit the effect of dog kidney extract on dog plasma. In general, after incubation with the extract, the action of the serum tends to inhibit the homologous extract and to accelerate or not to inhibit the action of the heterologous extract. In some cases there is noticeable a similar relation of the serum to the plasma. Dog serum tends to accelerate coagulation of goose plasma, irrespective of whether it is combined with dog kidney extract or chicken kidney extract. There exists then, in addition to the specific relation of serum to extract, also a specific relation of serum to plasma; if plasma and serum in combination with extract are heterologous the accelerating effect tends to predominate, while in the case of a homologous relation the inhibiting action tends to prevail. Thirdly, there exists the specific adaptation of the extract alone to the plasma; here the homologous combination produces coagulation most efficiently. All these factors interact in determining the results which are obtained with the different combinations of serum extract and plasma.

We may then conclude that 1, if sera, kidney extract and plasma are homologous or expressed differently if these substances are homoiogenous (derived from the same species) or if they belong to the same class of animals then serum inhibits the coagulation of the extracts on dog or goose plasma. 2. If these relations are heterologous, that means, if serum is obtained from one species or class of animals and extract and plasma are derived from a different species or class, the inhibition which serum otherwise would have exerted on the coagulating effect of extract is either diminished or changed into an accelerating action. 3. In those cases in which extract and plasma are obtained from different species or classes and one of these two substances is therefore homologous with the serum, while the other one is derived from a different species or class, then either the homologous relation between serum and extract or between serum and plasma, may determine an inhibition, or the heterologous relation between serum and either plasma or extract may determine an accelerating effect of the coagulating action of the extract on the plasma. In our experiments

it happened that the relationship of goose plasma to serum dominated over that of dog plasma to serum and likewise the relation of chicken kidney extract to serum dominated over that of dog kidney extract to serum. The relation between the dominating substance and the serum present in the mixture determined then whether an inhibition or an acceleration of the coagulation should take place.

These results taken together with our previous ones and with those of Burns, Scharles and Aitken make very probable the existence of a specific adaptation between the inhibiting substances in serum, on the one hand, and certain substances in extract and plasma, on the other hand.

The effect of heat on the extract. Heating dog kidney extract at 56° weakens its coagulating action on dog plasma, when acting alone, only very slightly or not at all; but the weakening effect may become evident when, after incubation of such extract with dog serum, the latter exerts its inhibiting action on the extract. Heating at 60° causes a more definite weakening of the effect of the uncombined extract; here also dog serum exerts a strongly inhibiting action, but in this case dog kidney extract alone is definitely weakened in its coagulating action on dog plasma. These effects of heating apply to diluted extract; with concentrated dog kidney extract, the weakening effect of heating at 60° is much less evident, but it can be demonstrated by incubation of the heated extract with dog serum. If dog kidney extract is tested with goose plasma, instead of with dog plasma, heating at 56° destroys its activity, although unheated dog kidney extract may cause rapid coagulation of goose plasma.

In the case of chicken kidney extract, heating at 56° does not apparently weaken the coagulating effect of the extract; heating at 60° weakens the concentrated extract only very slightly, it weakens somewhat more the dilute extract; but the inhibiting effect which goose serum exerts on extract, after incubation with it, proves that, after all, heating at 56° and still more so heating at 60° does cause a definite weakening of the extract. The inhibiting effect of goose serum is a graded one; it is more in evidence with extract heated at 60° than at 56°. Conditions here are similar to those stated above where incubation with dog serum brings out more clearly the weakening effect which heat exerts on the coagulating action of dog kidney extract. But we see that the depressing effect of heat is greater in the case of dog kidney extract than of chicken kidney extract. Furthermore the effect of heating depends also on the kind of the plasma on which the extract acts and on the kind of serum with which the extract is combined. Therefore, in the case of chicken kidney extract, the greater resistance to heating may be merely apparent.

In former investigations we had observed that heating of extract may cause a diminution in the inhibiting effect which, on incubation, serum exerts on extract. This effect came out especially if extract and serum

acted on fluorid plasma. With heparin plasma under the present conditions of experimentation, we have not observed a diminution in the inhibiting effect, which develops if the heated extract is incubated with serum.

We may conclude that not only the acceleration of coagulation of plasma, which takes place under the influence of tissue extract, but also the inhibiting effects, which occur if tissue extract is incubated with blood serum, depend on the species or class from which sera, tissue extracts and plasma are derived. Inhibition, as a rule, predominates over an accelerating effect of the mixture if serum and tissue extract or plasma are derived from the same species or class of animals. Inasmuch as there is no fact known which indicates that heparin would be able to manifest a specific adaptation to tissue extract of a certain class of animals, it becomes probable that the inhibiting substance which is present in the blood serum acts in combination with a protein on the coagulation of the plasma.

It is assumed by several investigators (F. Kraus and H. Fuchs, 14; A. Fischer, 15),—as it was formerly by Nolf—that the constituent of tissue extract which causes coagulation of the blood plasma is a combination of prothrombin,—identical with the prothrombin of blood serum,—and of certain lipoid substances acting as cytozym. Morawitz was of the opinion that the active constituent of the tissue acts as a kinase which changes the prothrombin or thrombogen into thrombin. We had suggested that tissuecoagulin, the active substance of tissue extract, acts directly on fibrinogen, inducing its coagulation and that in addition prothrombin of serum, as well as an inhibiting substance, which is present in the serum, may interact with substances in tissue extract in a way which differs specifically in the case of different species or classes of animals and depends upon the relation of these species or classes to each other. A direct interaction between tissuecoagulin and fibrinogen or plasma could be demonstrated in the case of invertebrate blood, and as we mentioned above, the great similarity which exists between the coagulation of invertebrate and of vertebrate blood, as far as the principles underlying both are concerned, as well as the results of certain experiments, which pointed to a direct interaction between tissue extract and plasma, when mixtures of serum and tissue extract were allowed to act on blood plasma, led to the conclusion that also in the coagulation of vertebrate blood tissue coagulin has the ability to act directly on fibrinogen (1a).

If we assume that the constituent of tissue extract, inducing or accelerating the coagulation of blood plasma, consists of a combination of a heat resistant lipoid and of prothrombin identical with that in serum, which latter is inactivated by heating at 56° for half an hour, then we would have to assume that a factor which is active in tissue extract renders the prothrombin present in the latter substance more heat resistant than

the corresponding substance in serum; or we would have to assume that the activity which remains in the tissue extract after heating it at 60° is due not to the tissue coagulin but to the lipoid substance acting as cytozym and we would moreover have to conclude that in tissue extract of chicken cytozym predominates more strongly than in mammalian tissue extract.

As far as suspensions of erythrocyte-stromata are concerned, it would be necessary to hold that lipoids (cytozym) are here the essential constituent which causes or accelerates coagulation, because the active principle present in them is more heat resistant than are the active substances present in tissue extracts. However, the specific adaptation which is demonstrable in the interaction between erythrocytes and plasma makes such an interpretation improbable.

Furthermore, before assuming that the agents causing or accelerating blood coagulation, which are present in serum and tissue extracts, are identical, it would first have to be shown that the prothrombin or thrombin of blood serum is specifically adapted to plasma or fibrinogen in the same way as tissue coagulin is specifically adapted to plasma or fibrinogen. As far as we know, a direct proof that this is so has not yet been given. However, as we pointed out on previous occasions, it is possible that the inhibiting substance, present in serum, covers up the specific adaptation of the thrombin.

There is another fact which has to be considered in this connection. While both prothrombin and tissue coagulin need calcium in order to cause coagulation of blood, the rôle of calcium does not seem to be the same in the action of these two substances. After calcium has once combined with prothrombin and cytozym to form thrombin, it is no longer needed for the action of the latter, whereas in the case of tissuecoagulin, the presence of calcium is required during the time when tissuecoagulin acts on plasma or fibrinogen (1a).

SUMMARY

We tested the effects of tissue extracts and blood serum of dogs and birds on the coagulation of dog heparin plasma and of bird plasma. A specific adaptation exists between the tissue extracts and the plasma, the effects which these substances exert on coagulation depending on whether the two substances, when combined are derived from homologous or heterologous species or classes of animals. In the case of blood sera a corresponding specific adaptation cannot be demonstrated.

If a mixture of serum and extract is added to plasma the serum causes an inhibition of the coagulating action which otherwise would be exerted by the extract alone, and this inhibition increases with increasing length of incubation of these substances, in case serum and extract and plasma

are homologous. If serum is derived from one species or class of animals and extract and plasma from another species or class, if therefore a heterologous combination is used, the addition of serum to tissue extract either causes a weakening of the inhibiting action or it may induce an acceleration of coagulation of the plasma. If serum is derived from the same class of animals as one of these two substances (extract or plasma), but differs in its origin from the other substance, then either the homologous or heterologous combination may predominate, determining either an inhibition of the coagulating effect in the first case and an acceleration in the second case.

There exists thus a specific adaptation between a substance in blood serum inhibiting the coagulation of blood plasma and substances in tissue extract and plasma, an effect which leads to the neutralization of the coagulating action of tissue extract. This specific inhibiting effect of the serum may be covered up, if the strength of the extract used is either too strong or too weak; in the former case the inhibiting serum action does not become manifest and, in the latter case, no coagulation occurs and it is therefore impossible to prove the existence of inhibiting effects. Through graded heating or dilution of the extract the strength of the extract can be regulated in such a way that the inhibiting effect of serum and its specific nature become clearly recognizable, heating and dilution of extract acting in the same direction.

Dog kidney extract is apparently not markedly affected by heating at 56°, but it is weakened at 60°. Bird kidney extract and stromata of erythrocytes are more resistant to heating than are mammalian kidney extracts. However, even in the case of these more heat resistant substances the weakening of these substances as the result of heating may become manifest, when extract is first incubated with serum previous to adding it to plasma, since under such conditions serum is able to inhibit the coagulating effect of the heated extract to a more marked degree than it would inhibit fresh extract.

The stroma of red corpuscles is specifically adapted to blood plasma of the same species of animals; but a specific adaptation between serum and erythrocyte-stroma cannot be clearly demonstrated, inasmuch as in combination with erythrocyte-stroma suspensions the inhibiting effect of serum is not definite.

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ON THE INHIBITING ACTION OF CATTLE AND SHEEP SERUM ON KIDNEY EXTRACTS OF CATTLE AND SHEEP[1]

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In preceding investigations (1) it has been shown that various blood sera, when incubated with tissue extracts, inhibit the coagulating effect, which the latter alone would exert on blood plasma, and that this inhibiting action increases with increasing incubation. The experiments furthermore pointed to the conclusion that in respect to this inhibiting effect a specific adaptation exists between serum and tissue extract and blood plasma. But there was an apparent exception to this made of interaction between blood serum and kidney extract inasmuch as Burns, Scharles and Aitken (2) had observed that cattle kidney extract when incubated with cattle serum and sheep kidney extract after incubation with sheep serum. instead of losing their coagulating effect to a large extent, lost only very slightly or not at all their ability to cause coagulation of blood plasma; mixtures of cattle serum and cattle extract retained their coagulating effect on dog heparin plasma even after an incubation period lasting as long as 80 minutes.

In the following investigations we wished to determine whether and under what conditions it might be possible to reverse the effects of the mixtures of cattle and sheep serum with their respective extracts and bring out the inhibiting, instead of the accelerating effect of blood serum on tissue extract also in this case.

1. The method of preparing the various reagents in our experiments were the same as those used in the experiments of Burns, Scharles and Aitken, except that the extracts were prepared from fresh instead of from dried, pulverized kidney. Finely ground sheep or beef kidey was mixed with an equal volume of 0.9 per cent NaCl solution; the coarser particles were removed by means of a fine sieve. The resulting suspension was kept for about three or four hours in the ice box and then used for the experiments. The dog plasma was prepared by the addition of 1 mgm. of heparin to 5 cc. of dog blood; the plasma was then obtained by centri-

[1] These investigations were carried out with the aid of a grant for research in science made to Washington University by the Rockefeller Foundation.

fuging. In each case 0.7 cc. serum and 0.3 cc. extract were mixed and this mixture was added to the plasma either at once or it was first incubated in the water bath at a temperature of about 37°C. for periods varying between 1 and 80 minutes. The quantity of heparin dog plasma used was in each case 1 cc.

First, we confirmed the observation of Burns, Scharles and Aitken, that addition of mixtures of beef or sheep serum with beef or sheep kidney extracts to dog plasma caused rapid coagulation of the latter notwithstanding previous incubation of serum and extract. We then considered it possible that by making more difficult and thus retarding the coagulation of the blood plasma, the inhibiting effect which serum exerts on the coagulating action of extract, following previous incubation of mixtures of serum and extract, might become manifest.

Three-tenths cubic centimeter of the kidney extract plus 0.7 cc. of a 0.9 per cent NaCl solution very rapidly caused coagulation of 1 cc. heparinized dog plasma. We began by adding various anticoagulant substances to such a suspension of kidney extract in 0.9 per cent NaCl solution. The quantity of the anticoagulant substance chosen was such that after its addition to the kidney extract the latter, when mixed with the blood plasma, required about 4 to 6 minutes in order to effect the coagulation of the plasma. In subsequent experiments this amount of anticoagulant substances was added to 0.3 cc. kidney extract. In further experiments 0.7 cc. of 0.9 per cent NaCl solution in this mixture was replaced by 0.7 cc. of the serum, the inhibiting effect of which we wished to test. For this purpose serum and extract together with the anticoagulant substance were incubated for various periods of time and at the end of the period of incubation 1 cc. dog heparin plasma was added to the mixture of serum, extract and anticoagulant. The anticoagulant substances used were: 8 per cent NaCl, 0.6 per cent K oxalate, 0.6 per cent NaFl, 0.6 per cent or 1 per cent Na citrate, and 0.5 mgm. or 1 mgm. heparin per 1 cc. of 0.9 per cent NaCl solution.

2. We found that under these conditions of experimentation the typical curves are obtained, showing an increase in the length of clotting time over that observed after addition of extract alone; this inhibition of coagulation is in general greater, the greater the length of the incubation period, although in different experiments the steepness of the curves may vary. Thus in some experiments we observed that after incubation for two minutes usually a rapid coagulation took place, while at a point between 20 and 40 minutes' incubation the coagulability may be lost entirely. In other instances, there was a steady increase in the length of coagulation time, until at last the mixture no longer caused clotting at all after addition of the plasma. However, exceptions occurred in which there was no marked increase in coagulation time with the lengthening of the incubation period. (See table 1.)

The absence of a lengthening of coagulation time was noticed especially in cases in which addition of 1 cc. of blood serum alone to the plasma, without admixture of extract, caused a relatively rapid coagulation of the plasma, usually within a period varying in different experiments between 10 and 90 minutes. On the other hand, if serum alone used in these quantities did not cause coagulation at all or only with a much greater delay, then the increase in inhibition with increasing length of the incubation period of mixtures of serum and extract was, on the average, quite pronounced. We may assume that in the former case the substance causing acceleration of coagulation was present in the serum in large quantity and that therefore even after long incubation with the extract this substance, perhaps after having first combined with a constituent in the extract, predominated strongly over the inhibiting substance in the serum, which presumably also enters into combination with a constituent

TABLE 1

BEEF SERUM—BEEF EXTRACT—1% SODIUM CITRATE			SHEEP SERUM—SHEEP EXTRACT—1% SODIUM CITRATE		
Time of incubation	Dog heparin plasma	Time of coagulation	Time of incubation	Dog heparin plasma	Time of coagulation
minutes	*cc.*	*minutes*	*minutes*	*cc.*	*minutes*
1	1.0	2	1	1.0	$\frac{1}{2}$
3	1.0	$1\frac{3}{4}$	3	1.0	5
5	1.0	$2\frac{1}{4}$	5	1.0	No clot
10	1.0	24	10	1.0	No clot
20	1.0	No clot	20	1.0	No clot
40	1.0	No clot	40	1.0	No clot
60	1.0	No clot	60	1.0	No clot
80	1.0	No clot	80	1.0	No clot

of the extract. This significance of the clotting power of the serum as such, for the result obtained, when we incubated serum and kidney extract, was noticed in all our experiments.

It is probably for the same reason that we found usually, although not in every case, a difference between the effect of serum A obtained by defibrinating blood directly after it had been received from the animal, and serum B prepared by allowing the blood to coagulate spontaneously and then removing the serum from the clot after the coagulated blood had stood in the ice chest overnight. Serum B when used alone as a rule caused more rapid coagulation of the heparin plasma than serum A; hence with mixtures of serum A and extract the typical inhibition curve was much more definite than with mixtures of serum B and extracts (table 2).

As to the efficiency of different kinds of anticoagulants only quantitative differences were found and it could be modified by varying the amounts of the anticoagulants. The results were the same irrespective of whether the anticoagulants exerted their inhibiting effect by inactivat-

ing calcium or whether like heparin they inhibited coagulation in some other way.

In further experiments it was determined that the results were also independent of the time at which the anticoagulant was added. The curve of inhibition was the same when the anticoagulant was mixed with the extract previous to the addition of the latter to serum or when it was added to the mixture after completed incubation; in the second case two minutes were allowed to elapse before the plasma was poured into the combination of serum, extract and anticoagulant.

We may conclude that the anticoagulant in delaying the coagulation of the blood plasma diminishes the effect of the factor, present in the mixture of serum and extract, which tends to accelerate the coagulation of the plasma, and conversely it favors the factor which tends to inhibit

TABLE 2

SHEEP SERUM B + SHEEP EXTRACT + HEPARIN SOLUTION (1 MGM. HEPARIN PER CC. PLASMA)		DEFIBRINATED SHEEP SERUM A + SHEEP EXTRACT + HEPARIN SOLUTION (1 MGM. HEPARIN PER CC. PLASMA)	
Time of incubation	Time of coagulation	Time of incubation	Time of coagulation
minutes	*minutes*	*minutes*	*minutes*
1	1	1	7
3	1	3	No clot
5	1	5	No clot
10	1	10	No clot
20	2	20	No clot
40	2¼	40	No clot
60	3	60	No clot
Serum B alone caused coagulation of dog heparin plasma in 40 minutes		Serum A alone did not cause coagulation of dog heparin plasma in 2 hours	

coagulation. The latter then increases in strength with increasing length of the period of incubation of serum and extract.

A curve showing increasing inhibition of coagulation, similar to the one caused by increasing the length of incubation of the mixture of serum and extract, is obtained by increasing the quantity of anticoagulant added to the extract. If then the serum is added and two minutes later the plasma, the coagulation time increases with increasing quantity of the anticoagulant and usually a steep rise in the curve occurs, if a certain amount of the anticoagulant has been exceeded. The coagulating effect predominates in the mixture of serum and extract, if only a small amount of inhibiting substance is added; but with the increase in the amount of inhibiting substance, the substance accelerating coagulation is no longer able to overcome the effect of the anticoagulant and now the inhibiting substance begins to predominate.

In experiments in which we combined sheep or cattle serum with the homologous or heterologous kidney extract, a specific adaptation between serum and extract, as indicated by the relative strength of the inhibiting or accelerating reactions, could not be observed; on the whole, the effects on the coagulation of the blood were similar irrespective of whether homologous or heterologous combinations were used, although in certain experiments a somewhat greater inhibiting effect of the homologous combinations seemed to be noticeable. It appears probable that if we use material from species which are relatively so nearly related as sheep and cattle the accelerating and the inhibiting substances which are active under these conditions interfere with each other in such a way that a definite specificity of the reactions cannot be observed.

Discussion. We may then conclude that in combinations of serum and tissue extract from sheep and cattle, in which under ordinary conditions there is a lack of the increase in inhibition of coagulation with increasing time of incubation, which is observed if we use extracts and sera from various other species, an inhibition can be obtained if we decrease the rapidity of coagulation of the plasma by adding various kinds of anticoagulant substances to the latter or to the extract. We may assume that under these conditions the effect of the inhibiting substance is made to predominate more and more over the accelerating substance with increasing time of incubation. This is presumably due primarily to the inactivation of the tissuecoagulin by the inhibiting substance of the serum which takes place in the mixture with increasing length of incubation.

However there may be a second factor which accounts for the increase of inhibition with increasing incubation. There takes place in all probability a diminution in the amount of active thrombin on the standing of the mixture of serum and extract; the rise in the curve indicating an inhibition in coagulation would then be due to the gradual loss of thrombin, as well as to the increasing inactivation of tissuecoagulin. As stated, this inhibiting effect is obtained irrespective of whether we add the anticoagulant substance to the mixture of serum and extract before incubation or after completed incubation, and also without regard to the kind of anticoagulant we use. The addition of calcium inactivating substances to the mixtures of serum and extract is effective even under conditions in which it may be assumed that the production of thrombin has been already accomplished. It appears probable that the inactivation of calcium affects unfavorably the effectiveness of the tissuecoagulin.

We also notice that the inhibiting effect of the addition of serum to the extract, on subsequent incubation of the mixture, is much more pronounced, if we use serum which has not had a chance to extract from the blood clot a large quantity of coagulation accelerating substance, or if we use a serum, such as that of the dog, in which naturally the inhibiting

substance predominates. In cases in which the increasing length of incubation of the mixture of serum and extract does not lead to an increase in the coagulation time, we may assume that the serum does not possess a sufficient amount of inhibiting substance or that the coagulating substance is present in the serum in so large a quantity that it overbalances the inhibiting substance which develops. Accordingly we have found formerly (3) that if we use fluorid plasma, the inhibiting effect of serum on tissue extract is rather slight, while it is considerable, when hirudin, pepton or heparin plasma is used. We may assume that in the former case, the coagulation takes place mainly under the influence of substances present in the serum, while in the latter case it takes place predominatingly under the influence of the tissuecoagulin which may be inhibited by substances present in the blood serum.

It is therefore probable that the inhibiting action of the serum is due mainly to its antagonizing effect on tissuecoagulin, but that the diminution in the amount of active thrombin, with increasing time of incubation of the mixture of serum and extract, may also be concerned in the increase in inhibition of the coagulation of the plasma which occurs under these conditions.

CONCLUSION

In mixtures of sheep or cattle serum and extract, which under ordinary conditions produce a rapid coagulation of heparin dog plasma, even after long continued incubation of the mixture, the typical curve of inhibition can be obtained, if the coagulation of the plasma is delayed through addition of either heparin or a calcium inactivating substance. This effect takes place, irrespective of the time at which the latter substances are added. The curve indicating increasing inhibition of coagulation of plasma, with increasing incubation of the mixture of serum and extract, is presumably due to the inactivation of tissuecoagulin by a substance in the serum, as well as to the destruction of thrombin, which progresses with increasing time of incubation.

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THE EXPERIMENTAL PRODUCTION OF ANEMIA IN DOGS BY MEANS OF A BLACKTONGUE-PRODUCING DIET

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It was reported in a previous publication (1) that ventriculin, a substance made from pigs' stomach and known to be efficacious in the treatment of pernicious anemia in man, could be used either to prevent or to cure the manifestations of vitamin B_2(G) deficiency in young albino rats. Strauss and Castle (2) and Castle and Rhoads (3) have shown that this vitamin deficiency in human beings may be related to pernicious anemia and sprue. Goldberger and Wheeler (4) extensively investigated the relationship between pellagra in human beings and blacktongue in dogs and concluded that the two conditions were analogous, being due solely to a lack of vitamin G (pellagra-preventive factor), the heat stable portion of the vitamin B complex. As far as we have been able to ascertain, however, they published no statement as to the development of anemia in their animals. Sebrell (5) described a condition in dogs on diets deficient in, or containing a marginal quantity of, vitamin B_2(G) in which a fatty infiltration of the liver was the characteristic finding at autopsy. The purpose of the present study is to determine the effect of a blacktongue-producing diet on the hematopoietic tissues of dogs.

MATERIALS AND METHODS. Six healthy appearing adult dogs were confined to our usual laboratory cages for several months' observation before they were restricted to the following modification of the blacktongue-producing diet of Goldberger, Wheeler, Lillie and Rogers (6).

Cornmeal	400 grams	Cottonseed oil	30 cc.
Cowpeas	50 grams	Cod liver oil	15 cc.
Casein (purified)[1]	95 grams	Salt mixture[2]	22 grams

Each of the six dogs was given daily one feeding of the above diet, being allowed to eat as much as desired. In addition, he received 2 cc. of a 50 per cent solution of ferric ammonium citrate each day. The diet plus the

[1] After the method of McCollum and, in addition, subsequent boiling in 95 per cent alcohol to inactivate any remaining traces of vitamin B_2.

[2] Prepared by the Harris Laboratories.

ferric ammonium citrate will be referred to hereafter as the basic diet. Two of the animals were given 75 grams of ventriculin per day each as a supplement to the basic diet. Red blood cell counts, hemoglobin determinations (Sahli acid hematin method), weighings, and reticulocyte counts according to the method kindly supplied us by Dr. Harry Goldblatt[3] were done each week. Often, if desired, the above determinations were made on all animals. Two of the four test animals died and blocks of tissue from all organs including bone marrow were fixed in 10 per cent formalin, sectioned and stained. The remaining two test animals were given 25 grams of yeast[4] daily for 10 days after they developed an anemia.

Observations. All of the dogs ate well during the first four weeks following the restriction to the basic diet. The red blood cell counts and hemoglobin determinations of the test and control animals rose slightly during the first two months. In the second month all the test animals lost their desire to eat the full diet and developed alopecia over the legs, shoulders, hips, scrotum, and around the anus. Ulcerations appeared over the points of contact and joints; the areas of both alopecia and ulceration increased slowly in size until the animal was treated. During the third month two of the four test dogs developed reddening of the mouth characteristic of blacktongue. These changes soon disappeared, however, without a change of diet, as often occurs in this disease. Each of the four test animals, regardless of the presence or absence of blacktongue, developed an anemia, the red blood cells and hemoglobin decreasing to 50 per cent of the initial value. The body weights of these four animals decreased progressively, the loss amounting to 20 per cent or 30 per cent before the end of the study. After the four test animals developed anemia, two of them were promptly remitted by the addition of yeast to the basic diet, the two which remained on the basic diet without yeast supplements died. Both animals which died showed partial paralysis of the hind legs on the last day, one had convulsions beginning two hours before death.

[3] Warm, clean glass slides are dipped in 0.3 per cent alcoholic solution of brilliant cresyl blue, tipped at an angle of 45° on filter paper, and allowed to dry. Freely flowing blood from the marginal vein of the dog's ear is drawn up to the 0.5 mark in a white cell counting pipette and diluted with a 0.3 per cent solution of brilliant cresyl blue in physiological saline to which has been added a small amount of potassium oxalate. The diluting fluid is drawn up to 11 and the pipette shaken. This technique is identical with that of making a white blood cell count. A small amount is expelled from the pipette, then a drop is placed on one of the prepared slides. A cover slip and then a piece of filter paper are placed upon the preparation. By the application of slight pressure, the depth of the smear and density of the cells may be readily controlled. The cover glass is rimmed with a melted mixture of beeswax and petrolatum which solidifies immediately. This prevents evaporation of the diluted blood, holds the cover slip firmly in place, and makes possible the use of an oil immersion lens for the examination. One thousand cells are counted.

[4] Yeast furnished through the courtesy of Dr. I. F. Harris, Tuckahoe, N. Y.

The daily hematological studies of the two animals given yeast (fig. 2) showed a prompt remission of the anemia. The type of hemopoietic

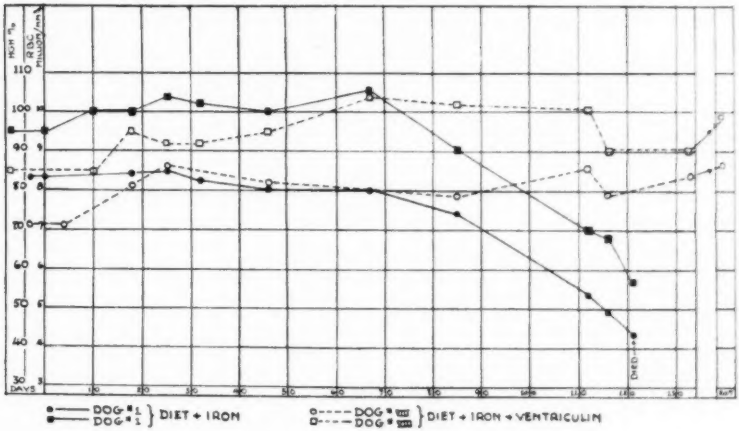

Fig. 1 shows a decrease in hemoglobin and red blood cell values in an animal on the basic diet alone and the maintenance of the normal values in another animal on the same basic diet but receiving large amounts of ventriculin.

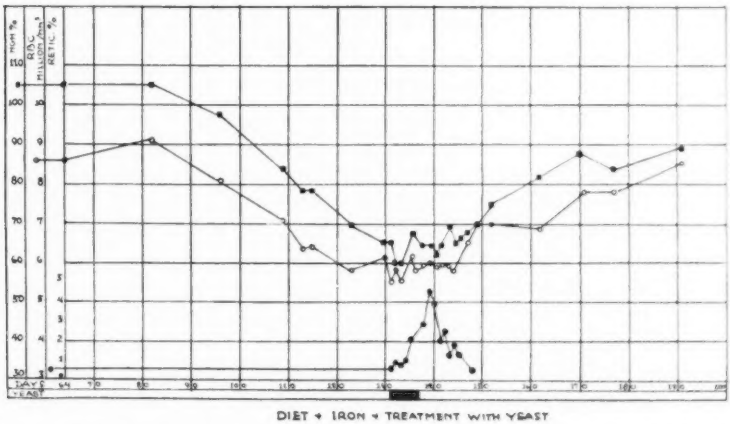

Fig. 2 shows the hemopoietic effect induced by adding yeast to the deficient diet after anemia has developed. Note that the reticulocytes are increased and are followed by subsequent increase in the number of red blood cells and amount of hemoglobin.

response which is seen in pernicious anemia after adequate therapy took place, namely: the reticulocyte count rose to a peak and quickly dropped,

following which there was a gradual rise of hemoglobin and red cells. Preceding this hemopoietic response, both animals showed an increase in appetite and the dermatitis improved. The two test animals which did not receive supplements of yeast continued to lose weight and became more anemic until their death in the fourth month of the study (fig. 1). The histopathological findings were a red and gelatinous bone marrow with corresponding microscopic findings. The viscera were examined but showed no significant pathological changes, either grossly or microscopically, other than fatty infiltration of the liver.

The two control animals receiving the ventriculin supplement ate well, gained weight, and did not develop dermatitis, diarrhea, blacktongue or anemia during the course of the study (see fig. 1).

Discussion. It has been shown in this experiment that the test dogs restricted to a blacktongue-producing diet developed dermatitis, diarrhea and anemia. In addition, two of the dogs developed transient blacktongue, while in the remaining two involvement of the central nervous system occurred. This condition appears to be similar to that described by Sebrell, although he published no observation on the blood and bone marrow. Previous investigators who have used either similar or identical diets to induce vitamin B_2 deficiency have not always reported uniform findings. For example, Underhill and Mendel (7) observed skin and oral changes; Goldberger and his co-workers described only oral and skin changes; Sebrell (5) found fatty infiltration of the liver; Miller and Rhoads (8) reported hematological and oral changes; while Zimmerman and Burack (9) stressed only nervous system involvement, but denied the presence of an anemia. The authors, on the other hand, have observed that two test animals had involvement of the skin, gastro-intestinal, nervous, and hemopoietic systems, while the remaining two test dogs had at least two of these systems affected. Our observations confirm those of Miller and Rhoads who maintain that a somewhat chronic deprivation of diet induces an anemia in some dogs, and those of Zimmerman and Burack who claim that animals restricted to such a basic diet develop neurological changes.

Furthermore, it has been shown by this study that large amounts of ventriculin protect dogs receiving the unbalanced diet against the deficiency state and also that anemia can be remitted by small amounts of yeast. The latter observation was likewise made by Miller and Rhoads (8).

No conclusive statement can be made as to the effects of such a deficiency diet on animals without taking into consideration the following factors: 1. Partial inanition due to loss of appetite in those animals restricted to experimental diets. 2. The varying degree of susceptibility of different animals to the development of one particular manifestation of this deficiency state. 3. Possible differences in chemical constituents of the re-

stricted unbalanced diets. 4. Variances in the quantity of food eaten by each animal on the same basic diet. 5. The length of time the animals are kept on the pellagra-producing diet. Not until such problems have been solved can we claim, as some observers are prone to do, first, that the lack of a single specific chemical substance causes pellagra or pernicious anemia in human beings; dermatitis in rats; blacktongue, anemia, dermatitis, and central nervous system involvement in dogs; and secondly, that vitamin B_2 deficiency, as judged by any one of the various manifestations following the continued ingestion of a markedly deficient diet, is a simple deficiency condition which develops following the lack of one chemical compound in the diet.

SUMMARY AND CONCLUSIONS

1. It has been shown in the present experiment that the loss of weight and development of anemia, dermatitis, and blacktongue in dogs can be prevented by the addition of large supplements of ventriculin to the basic diet and can be remitted by the addition of small amounts of yeast.

2. In view of our present inadequate information concerning the nature of the chemical substance or substances involved, it seems unwise to assume that the dermatitis, stomatitis, anemia, neuritis, and dementia of pellagra in human beings; and the dermatitis, blacktongue, diarrhea, anemia, and neurological involvement developing in dogs restricted to an unbalanced diet are all produced by the lack of the same specific chemical substance.

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THE REACTION OF CHRONIC SPINAL ANIMALS TO HEMORRHAGE

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Intense sympatho-adrenal discharge occurs in normal animals after a severe hemorrhage. The strong vasoconstriction due to activity of the sympathetic nerves (Cope, 1911; Pilcher and Sollmann, 1914; Meek and Eyster, 1921) aids in the restoration of blood pressure to a normal level. The adrenal medulla is also involved, for Bedford (1916), Tournade and Chabrol (1925), Saito (1928) and others have found that hemorrhage increases the adrenalin content of the blood. The increased adrenal discharge coöperates with the vasoconstrictor activity in restoring arterial pressure to a normal level. It also raises the blood sugar above normal (Saito, Kamei and Tachi, 1928; etc.) and, according to Cannon and Mendenhall (1914), causes a decrease in the clotting time of the blood. Because of their ability to compensate promptly normal animals can endure a relatively sudden loss of at least a third of their total blood volume.

Schlossberg and Sawyer (1933) reported that completely sympathectomized cats were incapable of compensating for mild hemorrhages of between 12 to 15 per cent of their blood volume. Although the arterial pressure rose slightly after hemorrhage, the rise was a few millimeters at most and never approximated the swift rise to the prehemorrhage level which is observed in normal cats. Slight additional loss of blood proved fatal to their sympathectomized animals, even though the total blood loss was far below that which would have seriously affected an animal with an intact sympathetic system.

In the series of experiments to be described, chronic spinal animals were subjected to sudden severe hemorrhages. It was thought that the response of these preparations would indicate whether such animals were virtually sympathectomized or could still show compensatory sympatho-adrenal activity. An ability to compensate would prove that the sympathetic system could be induced to discharge, as a result of hemorrhage and the accompanying low arterial pressure, despite being disconnected from higher centers of control.

METHOD. In the course of this work the effects of hemorrhages, varying from 10 to 25 per cent of the total blood volume, were observed in forty

chronic spinal cats and six spinal dogs. The animals were prepared by transecting the cord at the sixth cervical segment. Since the maximum response could be obtained in most cases ten days to two weeks after transection of the cord they were seldom kept for a longer period of time before being tested. The animals were fed a normal diet and care was taken to see that they did not become dehydrated during the post-operative period. They were catheterized daily. Occasionally intraperitoneal injections of saline were necessary in preventing dehydration because a high environmental temperature had to be used to preserve a normal body temperature.

The experiments were performed on anesthetized animals. Pentobarbital sodium (0.7 grain per kgm.) was commonly used but dial "Ciba" was twice employed. Four spinal animals were etherized and decapitated and two were decerebrated and the experiments continued without further anesthesia. These tests indicated that the anesthetic was in no way modifying the type of result obtained. The vagi were generally cut in the neck. A tracheal cannula was inserted. Arterial pressure was recorded from a carotid or femoral artery and blood samples were obtained from a branch of a femoral artery. In bleeding the animal a cannula was placed in a carotid artery and the blood allowed to flow freely into a graduate until the desired amount had been obtained. In calculating the severity of the hemorrhage the blood volume of the cats was considered to be 7 per cent of the body weight (Sherrington and Copeman, 1893; Meek and Gasser, 1918).

Results. *Restoration of arterial pressure.* It was found that spinal animals could restore their arterial pressure to the prehemorrhage level within a few minutes after a blood loss amounting to 15 to 25 per cent of their total blood volume (fig. 1). The compensation was slower and less effective than in the normal cat and comparable amounts of blood could not be withdrawn from the spinal animal without causing the blood pressure to drop to a fatal level. However, death was due to respiratory failure and if the spinal animals were given artificial respiration they survived the removal of surprisingly large amounts of blood. The ability of spinal preparations to compensate was influenced by the general physical condition of the animal.

The return of the blood pressure to a normal level suggested that the sympatho-adrenal system must have been acting, for, as previously stated, numerous investigators have shown that this system is activated by hemorrhage in normal animals and is responsible for their compensatory reactions. To test this, inactivation of the sympatho-adrenal system was performed and control experiments then executed. In those cases in which complete adrenal removal was desired the operation was performed acutely a few hours before the experiment. The sympathetic chains were removed as described by Cannon, Newton, Bright, Menkin and Moore (1929). Sym-

pathectomy was not considered to be complete unless each chain had been removed intact. Several weeks elapsed before these animals were used or cervical transection of the cord performed.

Denervation and complete removal of the adrenals did not seem to affect the compensatory ability of the spinal animals. The observation of Schlossberg and Sawyer (1933), that complete removal of the sympathetic chains greatly lessens the ability of animals to compensate for blood loss, was mainly confirmed in five sympathectomized cats which were otherwise normal. The cords of three sympathectomized animals were severed at the 6th cervical level. A few days after these operations the spinal sym-

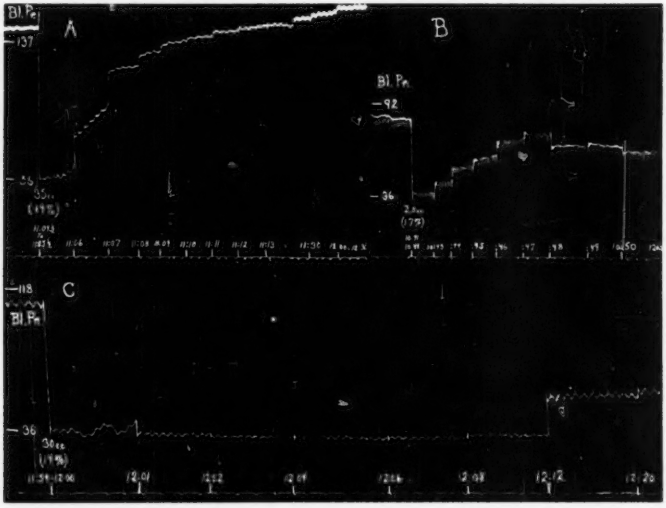

Fig. 1. The response of arterial blood pressure to hemorrhage in (A) normal cat, (B) spinal cat (6th cervical cord transection), and (C) spinal sympathectomized cat.

pathectomized animals were subjected to moderate hemorrhages and likewise failed to compensate for the drop in arterial pressure. However, these experiments on sympathectomized animals with and without their cords cut showed that such animals are not entirely devoid of compensatory ability. Although a hemorrhage of 10 to 15 per cent of their blood volume reduced the arterial pressure to a very low level and respiration failed, if they were given artificial respiration for several minutes the pressure eventually began to rise and finally returned sufficiently to insure an adequate blood supply to the centers and the animals began to breathe and eventually recovered. It is felt that blood dilution with restoration of blood volume must be the factor at work in producing this residual com-

pensation. No work, however, has as yet been done to support this belief and there is a possibility that some regeneration of the sympathetic fibers may have occurred in a few cases.

Changes in heart rate. It was found that even after transection of the vagi, hemorrhages occasionally produced retardation of the heart rate although an acceleration usually occurred if the cardio-accelerator nerves were intact or the adrenals present. In every case hemorrhage was followed by slowing of the heart in completely sympathectomized animals with vagi cut and from which the adrenals had been removed.

Vasoconstriction. In several experiments plethysmographs were placed upon the two hind legs, one of which had been completely denervated. In denervating the leg the abdominal sympathetic chain of that side was removed two weeks previous to the acute experiment to permit development of some intrinsic tone in the limb vessels. The remaining leg nerves were cut at the time of the experiment. Cattell (1923) has found that there is a decrease in leg volume when an animal is placed under conditions which stimulate general vasoconstriction. In seven experiments the decrease in leg volume following hemorrhage was always greater in the innervated leg than in the denervated member (fig. 2). The adrenals had been removed previous to the hemorrhage and this eliminated the chief source of humoral constrictor agents. That the difference in response of the legs was consistently present is the chief argument in favor of the occurrence of a vasoconstriction of nervous origin following the hemorrhages.

A slightly better method of testing for vasoconstriction of nervous origin was used in a few experiments (Pilcher and Sollmann, 1910). The arteries and veins of a hind leg were quickly tied off, leaving the nerves intact. The leg was then perfused with warm hemoglobin-Ringer solution (Amberson et al., 1934) at a pressure of 100 mm. Hg. A drop recorder was employed to measure the rate of flow of the fluid from the veins. These perfusion experiments showed only small differences between the innervated and denervated legs. The leg in which the sympathetic nerves had degenerated, due to a previous removal of the abdominal chain of that side, showed no change in the resistance to perfusion. In innervated legs, however, shortly after the hemorrhage the drops of perfusing fluid came less frequently and the original frequency did not return for about 45 minutes after the hemorrhage, when the blood pressure had been maintained at an approximately normal level for some time. These observations indicated a weak vasoconstriction or one confined to a small portion of the leg tissues. No adrenal factor could have been involved in these cases since the perfused member was entirely separated from the trunk circulation.

Since the vasoconstrictor nerves of the leg are relatively unimportant in comparison with the vasoconstrictor supply of the visceral organs, an

indirect method of studying vasoconstriction in the kidney was used. One kidney of a chronic spinal animal was completely denervated and the adrenals were removed. Cannulae were then placed in the ureters and the urine flow from the innervated and denervated kidneys was measured. The denervated kidney usually produced slightly more urine per minute than did the innervated organ. The urine flow was greatly lessened in both cases as a result of the low posthemorrhage arterial pressure but the

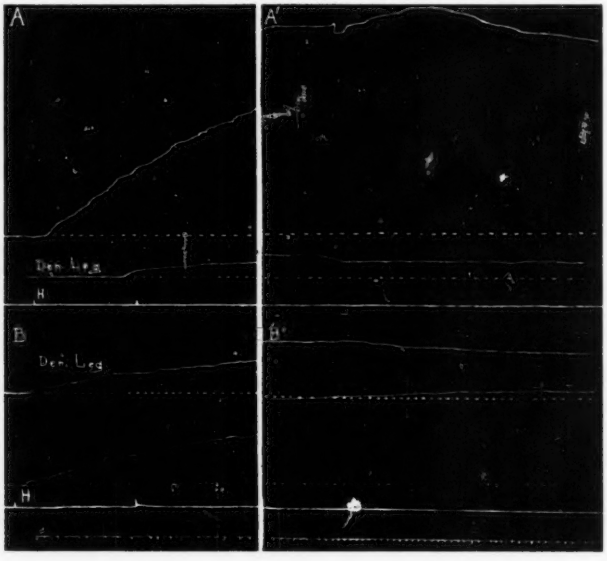

Fig. 2. Change in volume of denervated and normal hind legs resulting from hemorrhage. Dotted lines represent the base lines of the levers. Upward movement of lever indicates a decrease in volume. A-A'—Plethysmographic records obtained from a normal cat. Upper record innervated leg; lower record denervated leg. B-B'—Plethysmographic records obtained from the denervated hind leg (upper tracing) and a normal hind leg (lower tracing) of a spinal animal. H—Hemorrhage. Five minutes elapsed between A and A'; B and B'.

innervated kidney was affected to a much greater extent. The denervated organ began to produce more urine as the pressure began to rise and attained its original level of secretion within 30 to 45 minutes after the hemorrhage. The innervated kidney produced no urine at all for 20 to 30 minutes after the hemorrhage and it did not attain its prehemorrhage volume output during the hour and a half that the animals were kept following the hemorrhage (table 1). This again indicated that sympathetic vasoconstrictors are caused to act by the low pressures following hemor-

rhage. Somewhat similar experiments have been performed in normal animals by Bayliss and Fee (1930). They found that the decrease in urine flow following hemorrhage was in part due to a reflex inhibition of blood flow in the kidney.

Contraction of the nictitating membrane. The sensitized nictitating membrane has frequently been used as an indicator of sympatho-adrenal discharge (Rosenblueth and Cannon, 1932). Figure 3 presents the type of result obtained when this organ, sensitized by previous denervation, was employed to test for activity of the adrenals following hemorrhage. A contraction was obtained in both normal and spinal animals.

TABLE 1

DESCRIPTION OF ANIMAL	BEFORE HEMORRHAGE			HEMORRHAGE IN PER CENT OF TOTAL BLOOD VOLUME	IMMEDIATELY AFTER			5 MIN. AFTER			30 MIN. AFTER			1 HR. AFTER		
	Blood pressure	Drops per 5 min. interval			B.P.	Drops per 5 min. interval		B.P.	Drops per 5 min. interval		B.P.	Drops per 5 min. interval		B.P.	Drops per 5 min. interval	
		Innervated kidney	Denervated kidney			Inn.	Den.		Inn.	Den.		Inn.	Den.		Inn.	Den.
Normal-adrenals: In	182	12	20	17	100	2	5	138	7	12	142	6	13	140	8	14
Out	128	10	18	15	50	0	2	64	0	4	74	1	10	110	2	15
Spinal-adrenals: In	96	7	6	23	40	0	2	58	1	3	76	3	5	92	3	3
Out	86	10	12	15	44	1	6	40	0	2	60	0	5	80	2	10
Spinal-adrenals: In	76	24	26	11	40	2	4	62	10	15	70	8	12			
Out	70	8	9	10	36	1	3	40	0	2	52	1	6	80	5	8
Spinal-adrenals: Out	70	5	4	12	30	0	1	40	0	2	50	1	4	60	5	8
Decapitate-adrenals: Out	100	5	9	15	42	0	2	40	0	3	65	0	5	75	4	13

Changes in clotting time. A change in clotting time of the blood is not a very delicate or reliable indicator of adrenal discharge and activity of the sympathetic nerves but the results obtained were in agreement with the other tests for hemorrhage-induced activity of the sympathetic system. The blood did clot more swiftly following hemorrhages in spinal cats when the adrenals and sympathetic nerves were intact. The variations in speed of blood clotting were determined by the Cannon and Mendenhall (1914) technique.

Rise in blood sugar concentration. The sympatho-adrenal discharge which is involved in the compensatory responses of normal animals to severe hemorrhage produces a rise in blood sugar (Saito, Kamei and Tachi,

1928). This is likewise true of the spinal animals. Table 2 tabulates the effects produced by hemorrhages in a variety of preparations. The Folin-Svedberg (1930) method was followed in investigating blood sugar changes.

Site of origin of sympatho-adrenal activity. Denervation of the adrenals did not completely insensitize the gland to the conditions produced by a severe hemorrhage. It has been shown that the denervated adrenal gland will discharge adrenine as a result of asphyxiation (Cannon and Hoskins, 1911; Zwemer and Newton, 1928). It seems probable that the low post-hemorrhage blood pressures might have stimulated the denervated gland, possibly by cellular asphyxia. At any rate evidence of adrenal discharge was obtained. Slight contraction of the denervated nictitating membrane

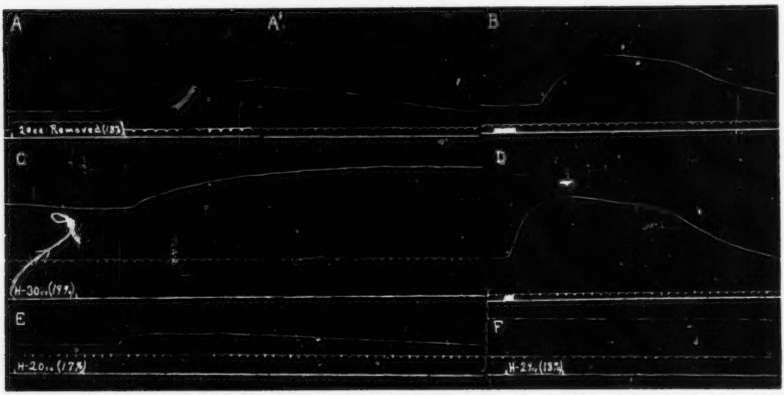

Fig. 3. Contractions of the denervated nictitating membrane following hemorrhage (H). A-A′ Spinal cat. Five minutes between A and A′. B-Intravenous injection of 0.5 cc. of 1:250,000 adrenalin solution into same spinal animal. C-Normal animal. D—Injection into same normal of 0.5 cc. of 1:250,000 adrenalin. E—Spinal sympathectomized animal with adrenals in (denervated). F—Spinal sympathectomized cat with adrenals removed.

occurred following hemorrhage in a few spinal animals with denervated adrenals, although it was never observed after complete removal of the glands. Heart rate, blood pressure, clotting time and blood sugar changes were not greatly affected by adrenal denervation but complete removal of the glands practically abolished them, especially in the absence of the sympathetic chains. In completely sympathectomized cats the adrenal glands had to be removed before all the signs of sympatho-adrenal discharge, normally following hemorrhage, were abolished.

The possibility that the low posthemorrhage blood pressures might have evoked the compensatory vasoconstriction and sympatho-adrenal activity through an effect on some peripheral mechanism was considered. All the

dorsal roots of three cats were cut between C-7 and L-5, and the cord was then transected at these levels thus eliminating any possibility of the sympathetic outflow being stimulated reflexly. Although the changes in heart rate following hemorrhage were much smaller than those observed in spinal animals with intact dorsal roots, the vasoconstrictor activity seemed to be equally good since the arterial pressure returned to normal quickly. Cutting both dorsal and ventral roots rendered the spinal animal as unable to compensate promptly as were sympathectomized animals. In all cases adrenals were removed acutely to prevent the complications resulting from a direct effect of the low pressures upon the gland. These last experiments indicated that the spinal cord is affected directly by the posthemorrhage

TABLE 2

The effect of hemorrhage on the blood sugar level

DESCRIPTION OF ANIMALS	NUMBER OF ANIMALS	AVERAGE BLOOD SUGAR IN MGM. PER 100 CC. BEFORE HEMORRHAGE	AVERAGE HEMORRHAGE IN PER CENT OF TOTAL BLOOD VOLUME	AVERAGE BLOOD SUGAR IN MGM. PER 100 CC. OF BLOOD				AVERAGE CHANGE IN MGM. PER 100 CC. OF BLOOD
				5 min. after hemorrhage	15 min. after	30 min. after	1 hr. after	
Normal: Control	5	135	18	165	194	119	178	+59
Spinal: Otherwise normal	10	105	13	119	132	130	103	+27
Decapitate: Otherwise normal	3	118	16	137	154	150	122	+36
Spinal: Adrenals denervated	3	110	14	128	130	138	113	+28
Spinal: Adrenals out	3	82	14	93	93	80	64	+11
Spinal: Sympathectomized; adrenals in	2	108	13	113	104	101	98	+5
Spinal: Sympathectomized; adrenals out	2	96	14	96	96	90	87	0

conditions and that the compensatory vasoconstriction, etc., originates there.

Discussion. The spinal cord even when disconnected from higher centers of control is capable of mediating some reflex activity of the sympathetic nerves and the adrenal medulla. Nociceptive stimuli elicit a marked rise in arterial pressure (Sherrington, 1906), blood sugar, heart rate, contraction of the denervated nictitating membrane and a diminished clotting time of the blood. These effects are the result of a reflex discharge of the sympatho-adrenal system (Brooks, 1933).

The results of this present work show that hemorrhage likewise produces strong sympatho-adrenal discharge in the spinal animal. It is felt that no one of the tests employed for the detection of activity of the sympathetic system would be completely satisfactory proof if considered alone. How-

ever, the large number of tests used and their complete agreement indicate that some compensatory vasoconstriction and medulliadrenal discharge occurs in the chronic spinal animal following hemorrhage. Until a study can be made of the fluid restoration following hemorrhage, in sympathectomized and spinal cats, and its effect on blood pressure, the relative importance of the nervous vasoconstriction and the fluid restoration, in producing the posthemorrhage rise in arterial pressure, cannot be judged.

Gammon and Bronk (1933) have located sensory endings in the abdominal mesentery which are sensitive to changes in arterial pressure. Although no physiological function has been reported for them it was thought that they or some other peripheral carotid-sinus-like mechanism might be responsible for the excitation of compensatory vasoconstriction following hemorrhage in spinal animals. Since transection of all the dorsal roots of the thoraco-lumbar cord failed to abolish the posthemorrhagic rises in blood pressure, even though the heart rate did not rise as in spinal animals with dorsal roots intact, it was concluded that the cord must be directly affected by the low blood pressure or an asphyxiation arising therefrom and that no peripheral mechanism is necessary. Cutting the dorsal roots, however, did not eliminate the possibility that the postganglionic neurons themselves were stimulated. When dorsal and ventral roots were cut no compensation occurred showing that the cord connections with the sympathetic ganglia are necessary and that the cord gives origin to the responses observed in the spinal animal.

SUMMARY

Spinal and decapitate cats can compensate for hemorrhages of between 10 to 25 per cent of their total blood volume. This compensatory ability is largely due to activity of the sympatho-adrenal system.

The activity of the sympatho-adrenal system is attested by a posthemorrhage vasoconstriction, a contraction of the nictitating membrane, a decrease in the clotting time of the blood and a rise in blood sugar level.

Removal of lateral sympathetic chains and adrenal medulla abolishes these compensatory responses.

Cutting the dorsal roots of the isolated thoraco-lumbar cord does not abolish the ability of the spinal animal to compensate and the blood pressure quickly returns to its normal level after a hemorrhage. Cutting the ventral roots in addition does abolish the compensatory activity. The origin of this response is in the cord.

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FORCES CONCERNED IN THE ABSORPTION OF THE CEREBROSPINAL FLUID

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It has long been known that the higher the pressure of injection, the more rapidly is an isotonic solution absorbed from the subarachnoid space. These early observations led to the idea that the absorption of cerebrospinal fluid was largely a process of filtration through membranes from a point of higher to a point of lower hydrostatic pressure. The finding that the venous pressure in the superior sagittal sinus is usually lower than the pressure in the subarachnoid space (6) seemed to lend support to the general hypothesis, but no convincing data setting forth the effective forces leading to this absorption of cerebrospinal fluid have been presented.

In considering the factors which might play a rôle in the process of absorption of cerebrospinal fluid, it became apparent that the colloid osmotic pressure of the blood and the hydrostatic pressure-difference between the subarachnoid pressure and the intracranial venous pressure should be the effective forces. The crystalloids of the blood and of the fluid are approximately the same in amount and nature, so that no effective pressures could be created by these substances. It therefore seemed feasible to attempt the determination of the total effective pressures playing a rôle in absorption of the fluid, for the osmotic pressure of the blood could be ascertained through use of appropriate celloidin membranes and the hydrostatic factor could be had by measurement of the subarachnoid pressure and of the sagittal venous pressure.

This experimental approach to the problem appeared possible as Mortensen and Weed (5) had recently devised a simple apparatus which permitted maintenance of any desired pressure within the subarachnoid space and at the same time permitted measurement of small amounts of fluid absorbed or extruded from this space. The apparatus consisted essentially of a pipette-reservoir system, with the foreign solution filling the pipette which was connected by tubing to a reservoir containing a kerosene-chloroform mixture of the same specific gravity as the foreign solution. With this apparatus attached to etherized dogs, measurements of the amount of Locke's solution absorbed at different pressures from the subarachnoid space were made. In these experiments by Mortensen and Weed the

pressure-increases were by multiples of the original pressure of the cerebrospinal fluid: the rates of absorption showed a linear relationship only in the lower pressure-range while at three or four times normal pressure a marked acceleration of the rate of absorption was noted.

Using this reservoir-pipette system, experiments on the absorption of foreign solutions were therefore undertaken on dogs etherized by intratracheal cannula connected to a Woulff bottle. The venous pressure in the superior sagittal sinus was determined by the method of Weed and Hughson (6), and the readings were made each minute throughout the period of experimentation. As in the previous observations the pipette-reservoir system was attached to the animal by a puncture-needle inserted through the occipito-atlantoid ligament into the subarachnoid space. The existing pressure of the cerebrospinal fluid for the individual dog was first determined by readings over a control period of 5 to 15 minutes, and then by opening the appropriate stop-cock the pipette-reservoir system was connected with the subarachnoid space. The pipette containing Locke's solution at body temperature was first thrown into the system and the reservoir set to maintain a pressure of 50 mm. saline above the normal pressure of the cerebrospinal fluid. After measuring the absorption of Locke's solution for 3 to 5 minutes at this pressure, the reservoir was slowly raised by successive increments of 50 mm. saline, and the rate of absorption determined for the solution at each of these established pressures for the same periods of time. Whenever the animal showed signs of increased intracranial pressure, the reservoir was not raised to any higher level: such signs usually made their appearance at 350 to 500 mm. saline above normal. But in the ordinary preparation the pressure could be increased the desired 400 mm. saline without appearance of any abnormality in breathing, movement, etc.

As soon as the rate of absorption of Locke's solution at the highest pressure was determined, the pipette-connections were altered so that the Locke's solution was shut off and the other foreign solution at body temperature introduced. A considerable period—10 to 15 minutes—was needed for the establishment of a new rate of absorption as the external system had to be washed free of the Locke's solution and the animal's subarachnoid space filled with the new foreign solution to the membranes of absorption. As soon as a constant rate of absorption was recorded, the reservoir was lowered by decrements of 50 mm. saline, with determination of the rate of absorption at each established level of pressure.

When the pressure in the reservoir-pipette system had been returned to normal or as soon as the second foreign solution was no longer absorbed, the puncture-needle was detached from the external system, and the cerebrospinal fluid, mixed with the foreign solution, collected from the subarachnoid space. By heart-puncture, blood was withdrawn, either

for whipping to secure serum or into oxalate (also citrate) solutions. The osmotic pressures of the blood samples and of the modified cerebrospinal fluid were determined by setting up at least two celloidin-tubes for each sample, in accordance with the method of Mayrs (3). The readings of these tubes were made in 20 to 22 hours, during which time they were kept at room-temperature; corrections for capillarity of the tube, for dilution of the blood with anticoagulant and for volume-changes occasioned by movement from the initial level were made.

Three protein-solutions were used and their absorption-rates contrasted with the Locke's solution. At body-temperature the viscosity of these solutions was such that they flowed from a burette at approximately the same rate as Locke's solution. The first of these protein-solutions was serum from another dog; the second was a gelatin-solution made up by dissolving 25 grams of gelatin in 400 cc. of Locke's solution. The third was a solution of pure casein, dissolved in M/15 phosphate buffer of pH 7.4.

With the data from the determinations of the colloid osmotic pressures of the blood and of the modified cerebrospinal fluid (obtained after the subarachnoid injection of the protein solution), it was possible to calculate the total effective pressures by adding the effective colloid osmotic value (colloid osmotic pressure of blood minus that of subarachnoid fluid) to the effective hydrostatic pressure which was determined by subtracting the sagittal venous pressure from the established subarachnoid pressure. Such a calculation is given in table 1 which presents the data from an adult male dog with absorption-rates for Locke's solution and for a gelatin-solution. When in a typical animal these absorption-rates per minute were plotted against the total effective pressure, a linear relationship for both the Locke's and the gelatin-solutions was apparent (fig. 1). A similar linear relationship was found to hold also for the casein-solutions and for the dog serum. The graph illustrating the absorption of the gelatin-solution was made by taking the average rates of absorption (as determined for intervals of 3 to 5 minutes at each of the maintained subarachnoid pressures) and also by taking the average of the sagittal sinus readings during the same periods. Such a use of average readings makes the linear relationship the more obvious: the points representing the individual average values fall therefore more nearly on the same straight line. The irregular spacing of the points along the abscissae is due to variations in the sagittal venous pressures as the subarachnoid pressures were raised and lowered by equal steps.

In addition to the experiments dealing with the comparative rates of absorption of Locke's solution and protein-solutions, a number of observations were made for comparison of the rates of absorption of simple non-

TABLE 1

C.S.F. PRESSURE	SAGITTAL VENOUS PRESSURE	C.S.F. MINUS SAGITTAL PRESSURE	COLLOID OSMOTIC PRESSURE BLOOD SERUM	COLLOID OSMOTIC PRESSURE MODIFIED C.S.F.	COLLOID OSMOTIC PRESSURE BLOOD MINUS SOLUTION	TOTAL EFFECTIVE PRESSURE	ABSORPTION RATE PER MINUTE
Locke's solution							
mm. saline	*mm. saline*	*mm. saline*	*mm. saline*	*mm. saline*	*mm. saline*	*mm. saline*	*cc.*
250	149	101	312		312	413	0 02
300	154	146	312		312	458	0 06
350	154	196	312		312	508	0 10
400	148	252	312		312	564	0 14
450	146	304	312		312	616	0 19
500	145	355	312		312	667	0 23
550	155	395	312		312	707	0 27
600	180	420	312		312	732	0 28
Gelatin solution							
300	137	163	312	98	214	377	0 003
350	150	200	312	98	214	414	0 03
400	144	256	312	98	214	470	0 07
450	135	315	312	98	214	529	0 11
500	145	355	312	98	214	569	0 15
550	152	398	312	98	214	612	0 18
600	149	451	312	98	214	665	0 23

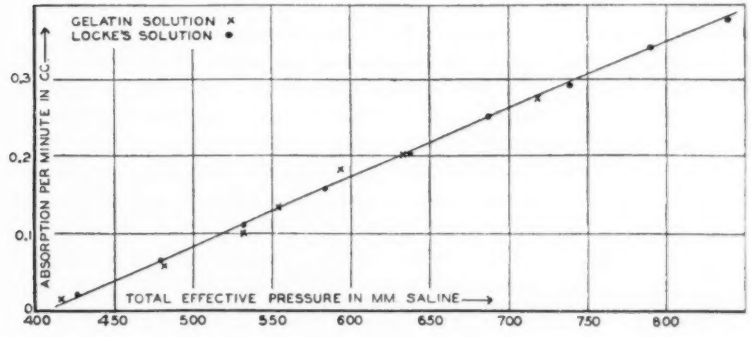

Fig. 1. Etherized dog. Absorption from the subarachnoid space of Locke's solution and of a gelatin-solution, under conditions of maintained pressures. Total effective pressure composed of hydrostatic pressure (subarachnoid pressure minus sagittal venous pressure) plus colloid osmotic pressure-difference between blood and cerebrospinal fluid (as withdrawn from subarachnoid space after injection of protein-solution). Points plotted are average readings.

isotonic solutions with Locke's solution. In the first group of these experiments, the rate of absorption of Locke's solution was contrasted with that of distilled water: surprisingly enough, the two solutions were found to be absorbed at exactly the same rate. Plotting these average rates of absorption against the hydrostatic pressure of the subarachnoid space minus that of the sagittal sinus (the colloid osmotic pressure of the blood adding merely a constant) gave a linear relationship. And when the subarachnoid absorption-rate of Locke's solution was contrasted with that of a twice-normal Locke's solution (in which double quantities of the salts were added to the same quantity of water), identical rates were found. Here again a linear relationship between absorption-rate and effective pressure was apparent. The explanation for these extraordinary results with distilled water and with twice-normal Locke's solution is not clear: it may be that such solutions within the subarachnoid space are quickly rendered isotonic.

These findings that the rates of absorption of an isotonic crystalloid solution (Locke's) and a protein-containing solution, when plotted against the total effective pressures, are described by the same straight line would seem to indicate that the factors assumed to make up the "total effective pressure" are actually the ones responsible for the process of absorption of cerebrospinal fluid. In spite of all of the difficulties and shortcomings of the methods for the determination of colloid osmotic pressures (cf. Meyer, 4; Landis, 2, et al.), the data when used as an average of two determinations seem adequate. While certain observers (Bonsmann and Brunelli, 1) have found that during anesthesia the colloid osmotic pressure of the blood changes, control observations under the same conditions as these experiments have given no indication that this osmotic pressure changes to any appreciable degree during the period of etherization (75 minutes). Were the colloid osmotic pressure of the blood inconstant during the period of experimentation, it would be impossible to detect any linear relationship for either Locke's solution or for the protein-solution.

These experiments indicate that the rate of absorption of a protein-solution from the subarachnoid space is slower than that of an isotonic crystalloid solution. The effective colloid osmotic pressure of the blood is diminished by the amount of the colloid osmotic pressure of the cerebrospinal fluid, here mixed with the protein-solution: the passage of fluid (water plus crystalloids) through the absorbing membranes is therefore retarded. It would seem reasonable to assume that protein does not leave the subarachnoid space in appreciable quantity during the short period of introduction of the foreign solution (30 minutes). The regularity of the experimental data indicate that there is no material change of protein-concentration in the subarachnoid space during the period of measurement. This constancy seems to be a function of the total quantity of the protein-solution in that space (relatively large at high pressures apparently) and of the amount of

water abstracted from the protein-solution by absorption. The experiments were apparently executed rapidly enough for the amount of the absorption of water to make no detectable difference in the protein-concentration.

From an analysis of the data here presented, it seems fair to conclude that the total effective force actuating the normal process of absorption of cerebrospinal fluid is compounded of the colloid osmotic pressure of the blood plus a hydrostatic factor derived from the difference in subarachnoid pressure and the intracranial venous pressure.

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THE SECRETORY METABOLISM OF THE SALIVARY GLANDS

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Various investigations suggest that the energy metabolism of the salivary glands is very similar to that of muscle. While working, the glands remove sugar and oxygen from the blood and liberate carbon dioxide (Anrep and Cannan, 1922, 1923; Barcroft, 1901), lactic acid and phosphates (Bergonzi, 1931). Cyanides and monoiodoacetic acid have about the same effects, qualitatively, as in muscle (Ferrari and Höber, 1933). It might therefore be expected that analysis of the gland itself after activity would show the same changes as does fatigued muscle. But Himwich and Adams (1930) analyzed active and resting glands for lactic acid, and were unable to demonstrate a consistent increase in stimulated glands, nor in the blood coming from them. They stimulated the chorda tympani until the gland was exhausted. Their results are, however, highly variable, and it is possible that in some of their experiments considerable removal of lactic acid may have taken place before analysis, as they did not freeze the glands on removal from the animal.

These results are in contradiction to the indications in the earlier literature that salivary gland metabolism is similar to that of muscle. The failure of Bergonzi and Bolcato (1931) to find a decrease in glycogen on stimulation of the gland also does not agree with this assumption. And furthermore, no investigation has been made on what would appear to be the most crucial question—whether or not there is a decrease in the amount of creatine phosphate in the gland during activity. This direct approach to the question has been attempted, along with a more detailed study of the changes in glycogen and lactic acid, with a view to obtaining a direct comparison of the activity metabolism of the salivary gland with that of muscle. Forty-seven experiments of various types were performed, and determinations made of the glycogen, lactic acid, and creatine phosphate content of resting and exhausted glands.

METHOD. Dogs under amytal anesthesia were used. Amytal depresses salivary secretion somewhat (Stavraky, 1931), but this condition was considered to be preferable to the frequent spontaneous secretion of all salivary glands which occurs with ether.

The two submaxillary glands were freed from their capsules by blunt

dissection, care being taken not to interfere with their blood supply. The duct of the gland chosen for stimulation was cannulated.

In order to get maximum activity from the gland, in most experiments both sympathetic and parasympathetic nerves to the gland were stimulated simultaneously by placing the electrodes on the tissue just under the duct where it leaves the gland, and maximal stimulation with faradic current continued until the secretion of saliva had ceased or become very slow. Stimulation was discontinued and the exhausted gland quickly removed and frozen in liquid air. Then the unstimulated gland was removed and frozen and both glands were then analyzed.

The gland chosen for stimulation was alternated from one experiment to the next, so that an equal number of right and left glands appears in each stimulated, as in each control, series.

Glycogen was determined by a modified Pflüger method, as described by Smith and Visscher (1930), hydrolysis being carried out with sulfuric acid instead of hydrochloric in order to allow the use of the Folin-Wu sugar technique. The gland sample was broken up and weighed while still frozen, and thawed in the presence of hot 40 per cent KOH.

Lactic acid was determined by the method of Friedeman, Cotonio, and Shaffer (1927) on a Folin-Wu filtrate prepared from the gland as follows: The gland, still frozen, was thoroughly pulverized in a mortar cooled with liquid air. A sample was weighed and reduced to a paste by grinding with 10 per cent sodium tungstate as it thawed. Appropriate amounts of water and $\frac{2}{3}$ N sulfuric acid were added, and after precipitation was complete, precipitate and fluid were thoroughly mixed by further grinding in the mortar, and then the mixture was centrifuged and filtered. Approximately twice as much tungstate and acid are required for complete precipitation as for blood, the water being reduced accordingly, to give the usual one to ten dilution of the tissue.

Creatine phosphate was determined by the method of Lohmann (1928).

Results. All the experiments on lactic acid and glycogen changes are summarized in table 1. Thirteen experiments were performed in which all the nerves to the gland were stimulated and the glands analyzed for glycogen and lactic acid.

In every experiment there was a sharp decrease in glycogen in the stimulated gland compared to the resting one. On the average, there was 41 per cent less glycogen in the stimulated gland.

Of the ten experiments in which lactic acid was determined, none showed more lactic acid in the stimulated gland; in the tenth the amounts were about equal. In three cases the lactic acid determination was lost. In some cases the increase in lactic acid in the stimulated gland as compared to the resting was striking; as high as 262 per cent in one case.

For the percentage decrease of glycogen on stimulation, the standard

deviation is 3.60 per cent. This gives a value of 1.04 per cent for the standard error. The mean decrease in glycogen, 41.4 per cent, is forty times its standard error, and is therefore of undoubted significance.

TABLE 1

PER CENT GLYCOGEN			LACTIC ACID, MGM. PER CENT			CUBIC CENTIMETER SALIVA BEFORE EXHAUSTION
Control gland	Stimulated gland	Per cent decrease	Control gland	Stimulated gland	Per cent increase	
Stimulation of all nerves to gland						
0.24	0.17	29.2				68
0.30	0.18	40.0	46	45	−2	26
0.32	0.15	53.2				20
0.34	0.25	26.5	36	130	262	7
0.24	0.10	58.3	36	90	150	29
0.26	0.15	42.3				14
0.30	0.21	30.0	37	54	46	28
0.30	0.13	56.7	31	93	200	5
0.32	0.13	59.3	25	73	192	12
0.32	0.14	56.2	41	77	88	25
0.35	0.24	31.4	20	40	100	28
0.31	0.21	32.3	28	33	18	29
0.29	0.22	24.1	27	94	246	5
Av. 0.30	0.18	41.4	33	73	128	23
Chorda tympani stimulation						
0.26	0.25	3.9	23	31	34.8	14
0.32	0.26	18.7	27	29	7.4	7
0.28	0.21	25.0	37	42	13.5	12
0.36	0.30	16.7	46	50	8.7	6
Av. 0.31	0.26	16.1	33	38	16.1	9.8
Sympathetic stimulation						
0.33	0.28	15.2	19	24	26	0.3
0.24	0.20	16.7	24	24	0	0.4
0.23	0.11	52.2	53	110	108	1.0
0.23	0.20	13.0	48	58	21	0.3
0.32	0.21	34.4	25	68	172	0.8
Av. 0.27	0.20	26.3	34	57	65	0.6

The standard deviation of the lactic acid differences is 38.24 per cent, and the standard error of the mean difference is 9.41 per cent. The mean difference, 128 per cent, is over thirteen times the standard error.

It seems certain, therefore, that on stimulation of all the nerves to the gland there is a large and genuine decrease in glycogen, accompanied by a

somewhat more variable but undoubted increase in lactic acid. Since the gland was not poisoned in any way, and presumably had an intact blood supply, it is to be expected that more or less of the lactic acid formed would be either oxidized or removed by the blood. It was therefore considered sufficient that in the majority of cases a significant amount accumulated in the gland during stimulation; in those cases where an increase was absent or relatively slight, it is presumed that the processes of removal and oxidation were able to keep up with formation.

As noted above, Himwich and Adams found no consistent changes in lactic acid content on chorda stimulation. This type of stimulation was employed in four experiments. The rate of secretion evoked is smaller, and the chemical differences noted are also less. The standard deviation of the percentage decreases in glycogen is 3.83 per cent, the standard error 2.21 per cent. For the percentage increases in lactic acid, the standard deviation is 5.53 per cent, the standard error is 3.19 per cent. The glycogen decreased seven times the standard error, the lactic acid increased five times the standard error. Compared with the previous series the differences are only 15 per cent as great in lactic acid, and 40 per cent in glycogen changes. The great vasodilatation caused by chorda tympani stimulation may wash away more of the liberated lactic acid. The greater relative change in glycogen is in harmony with this view, and there seems no reason to doubt that qualitatively, at least, chorda stimulation causes the same type of carbohydrate changes as does the other type of stimulation.

Since the stimulation of the chorda alone produced changes strikingly smaller than those given by stimulation of all the nerves to the gland, it was decided to perform some experiments with sympathetic stimulation. The electrodes were applied to the sympathetic trunk where it leaves the vagus high in the neck. Such stimulation gives greater changes in glycogen and lactic acid than does chorda stimulation, but qualitatively the results are the same. The changes are significant: the standard deviation of the glycogen decreases is 6.74 per cent, the standard error 3.37 per cent, and the average decrease, 26.3 per cent, is nearly eight times the standard error. The standard deviation of the lactic acid increases is 28.80 per cent, the standard error 14.40 per cent, and the average increase, 65 per cent, is four and a half times the standard error.

The next series of experiments concerned the creatine phosphate content of the glands. Preliminary experiments showed that, in contrast to the glycogen and lactic acid content, there is likely to be some variability in the amount of creatine phosphate present in pairs of glands from the same animal. Therefore a control series of some length, in which neither gland was stimulated, was run. The average of sixteen pairs of glands showed that, in spite of a certain number which contained different amounts in the right and left gland, the average creatine phosphate content of the right

submaxillary gland is equal to that of the left—an average of 3.7 mgm. of creatine phosphate (as P) per 100 grams of gland. The standard error is 1.42 per cent, or 0.05 mgm.

Thus, in spite of some random variability in creatine phosphate content of paired glands as used in these experiments, the effects of stimulation of the gland can be determined if the differences produced are regular and large.

Nine experiments were performed in which one gland was stimulated to exhaustion, all nerves to the gland being excited. There was a marked decrease in creatine phosphate phosphorus in each of the stimulated glands. The results are given in table 2.

The standard deviation of the percentage decreases is 8.38 per cent, the standard error 2.96 per cent. The average decrease in creatine phosphate (as P), 63.4 per cent, is twenty-one times the standard error.

TABLE 2

Creatine phosphate content of stimulated and control glands, as P

CONTROL, MGM. PER CENT	STIMULATED, MGM. PER CENT	PER CENT DECREASE
3.3	1.8	45.5
2.7	1.0	62.9
7.8	5.9	24.4
3.1	1.7	45.2
4.1	0.5	87.8
2.3	1.4	39.1
3.2	0.9	71.8
3.1	0.2	93.6
1.9	0.0	100.0
Av. 3.5	1.5	63.4

Discussion. The results clearly show that the submaxillary gland obtains energy for its secretory mechanisms from the same sources as does striated muscle in contraction. Creatine phosphate is broken down, glycogen decreases as lactic acid accumulates.

In order to accomplish proportionate chemical changes a more prolonged stimulation of the gland is necessary than is the case with isolated muscles. Recovery processes, in the presence of adequate blood supply, are apparently almost able to keep up with catabolic events. Stimulation of the chorda tympani alone produces the slightest changes of the whole series, which is probably the reason that Himwich and Adams (1930) failed to find a significant increase in lactic acid, and that Bergonzi and Bolcato (1931) were unable to detect any breakdown of glycogen. These investigators stimulated the chorda tympani.

It is rather surprising that sympathetic secretion should prove to be so

"expensive" as to glycogen usage; the production of less than one cubic centimeter of sympathetic saliva gave a somewhat larger decrease in glycogen than did the secretion of around 10 cc. of saliva from chorda tympani stimulation. This is possibly due to the fact that during sympathetic stimulation the gland is working with a diminished blood supply, which would interfere to some extent with recovery processes; while during chorda tympani stimulation, there is an increased blood supply, and an opposite effect on recovery.

The breakdown of creatine phosphate during stimulation of the gland is in accord with the findings of Bergonzi (1931) that phosphoric acid is liberated into the blood by the stimulated gland, and with the observations of Ferrari and Höber (1933) that monoiodoacetic acid increased this production of phosphoric acid.

SUMMARY AND CONCLUSIONS

1. Simultaneous stimulation of sympathetic and parasympathetic nerves to the submaxillary gland of the dog causes a decrease in the glycogen, an increase in the lactic acid, and a decrease in the creatine phosphate in the gland.

2. The average decrease in glycogen due to exhaustive stimulation of this type is 41.4 per cent; the average increase in lactic acid is 128 per cent; the average decrease in creatine phosphate phosphorus is 63.4 per cent.

3. Exhaustive stimulation of only the parasympathetic nerves to the gland causes an average decrease in glycogen of 16.1 per cent, and an average increase in lactic acid of 16.1 per cent. These changes are much smaller than those from simultaneous stimulation of all nerves to the gland.

4. Exhaustive stimulation of only the sympathetic nerves to the gland causes an average decrease in glycogen of 26.3 per cent, and an average increase in lactic acid of 65 per cent. These changes are intermediate in magnitude between those produced by stimulation of all the nerves and by stimulation of the parasympathetics alone.

5. The lesser changes during parasympathetic than sympathetic stimulation may be due to vasodilatation during the former, and a consequent increase in the rate at which recovery processes involving oxidation can occur. The secretion of sympathetic saliva is probably only apparently more "expensive," and seems to be so because of vasoconstriction during sympathetic stimulation interfering with recovery processes.

6. Since the breakdown of creatine phosphate and glycogen, with a simultaneous accumulation of lactic acid, are found to occur during salivary secretion as well as during muscular contraction, it is suggested that the creatine phosphate-glycogen mechanism may be a rather general one for the release of energy in various tissues where work is done.

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THE EFFECT OF EPINEPHRINE ON ARTERIAL AND VENOUS PLASMA SUGAR AND BLOOD FLOW IN DOGS AND CATS

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Previous experiments have shown that the minimal rates of intravenously injected epinephrine which cause hyperglycemia are 0.0015 mgm. per kilo per hour in man, 0.003 in rabbits and 0.012 in rats (1). These rates of injection are smaller than those required to increase blood pressure. Similar figures have not so far been established for dogs and cats. According to data published recently by Soskin, Priest and Schutz (2), dogs would appear to be particularly insensitive to epinephrine, since a rate of injection of 0.06 mgm. per kilo per hour produced hardly any hyperglycemia. Such a rate of injection is generally pressor, so that the dog would also differ from the species mentioned above in that a larger dose of epinephrine is required to give a blood sugar than a blood pressure response. These considerations made it desirable to investigate the action of epinephrine in carnivorous animals. The exchange of sugar, lactic acid and water between blood and the tissues of the leg was determined by analysing arterial and venous blood for these constituents, and blood flow was recorded in muscle or in the whole leg. The data permitted a critical examination of the significance of the arterio-venous blood sugar difference as a measure of sugar utilization by the tissues.

EXPERIMENTAL. Cats and dogs after a fasting period of 18 hours, were anesthetized by an intraperitoneal injection of amytal or nembutal. During the experiment additional doses of these drugs were injected as needed so as to maintain an even anesthesia. Body temperature was kept as constant as possible by heating pads. In some experiments performed in the summer months it was necessary to cool the animal by an electric fan in order to prevent a rise in body temperature.

Blood pressure was recorded in the carotid artery by means of a mercury manometer. Epinephrine was injected intravenously at a constant rate. A ratchet operated by a suction pump and provided with suitable electric contacts was timed by a Bowditch clock, the ratchet pushing the plunger of a 50 cc. syringe each time the clock made an electric contact. Each contact was recorded on the smoked drum by means of a signal magnet.

Generally 20 cc. of the epinephrine solution were injected per hour. The latter was prepared (from the 1:1000 Parke-Davis and Co. stock solution by means of physiological salt solution) shortly before needed and was protected against inactivation in vitro by addition of 2 cc. of serum or of 20 mgm. of glutathione per 100 cc. of solution. In 2 experiments 20 mgm. of alanine per 100 cc. was added as anti-oxidant.

The thermo-electric device described by Gibbs (3) was used as an index of the rate of blood flow. This consists of a constantan-iron thermocouple which may be inserted in a muscle or placed in the sheath of the femoral vessels. One junction is heated electrically to a temperature about 2° above that of the animal. The thermocouple is connected to a galvanometer[1] which records the changes in E.M.F. produced by the flow of blood past the heated junction. An in vitro calibration serves to establish the relation between rate of flow and the deflection of the galvanometer. During the experiment the spot of light on the galvanometer scale is followed by a pointer which transmits the movements, magnified about 2.5 times, to a smoked drum. Since a difference in temperature of 1°C. between the two junctions corresponded to 50 microvolts or to a deflection of 33 mm., a change in temperature of 0.02° could be measured.

Fluctuations in the temperature of the animal are compensated because both junctions are located in close proximity (about 1 cm. apart) in the same tissue. After the two junctions had been placed in the tissue and before heat was applied to one junction, it was ascertained whether or not the two junctions were at the same temperature. If this was not the case, the position of one junction was changed until equal or nearly equal temperature was obtained.

Control experiments consisted in testing the thermocouple with the heating current alternately turned on and off. Procedures which were known to lead to a change in blood flow, while causing a deflection of the galvanometer with the heat turned on, had no effect with the heat turned off. In several experiments, in order to test the sensitivity of the blood flow recorder, the thermocouple was placed alongside the femoral vessels in the groin and the saphenous vein was clamped or blood was withdrawn from it at varying rates. Thus clamping the saphenous gave a deflection of the galvanometer corresponding to 9.5 microvolts, withdrawing blood at 13 cc. per minute to 2.6 and at 6 cc. per minute to 1.5 microvolts, so that with a rate of blood flow of 40 cc. per minute through the leg, a change in blood flow of 10 per cent could be detected.

Blood was withdrawn from an artery (brachial or carotid) and from the unobstructed femoral vein as nearly simultaneously as possible (within 1

[1] The galvanometer (type R of Leeds and Northrop) had an internal resistance of 561 ohms, a period of 3.3 seconds and gave a deflection of 1 mm. at a distance of 1 meter per 1.5 microvolts. A potentiometer served as a measuring instrument and as a means of locating the spot of light on the galvanometer scale.

minute). The plasma was separated by centrifugation in capped tubes. When oxalate was used as an anticoagulant, care was taken to use equal amounts for the arterial and venous blood, so as to equalize the small water shifts between corpuscles and plasma which are apt to occur. In a number of experiments heparinized plasma was used and in others oxalated whole blood was analyzed, with no apparent difference in results. One cubic centimeter of plasma or whole blood was precipitated with copper sulfate and sodium tungstate (Somogyi, 4) and analyzed in duplicate by means of the Shaffer-Somogyi (5) reagent containing 1 gram KI. The glassware used was carefully standardized. The titration differences of duplicates were generally less than 0.02 cc. of 0.005 N thiosulfate. One-half cubic centimeter of plasma or whole blood was measured with an Ostwald pipette and placed in a tared weighing bottle containing a fluted filter cone. The difference in weight before and after drying to constant weight at 105° gave the water content of the sample. Lactic acid was analyzed by means of the apparatus described by Wendel (6).

Changes in blood flow. In unanesthetized rabbits it is possible to increase blood sugar and blood lactic acid with rates of injection of epinephrine $\frac{1}{10}$ of those required to raise blood pressure (7). It was soon found that in amytalized dogs and cats the minimal pressor and minimal hyperglycemic rates of injection are not so far apart as in the rabbit. It seemed inadvisable, therefore, to rely on blood pressure alone and it was necessary to determine blood flow as well.

A number of experiments (some of which are shown in figs. 1 and 2) were performed with varying rates of injection in order to find out what changes in blood flow in muscle and in the whole leg might be expected under these conditions. In figure 1 a with no heat applied to the junction (this being the control procedure mentioned earlier) and with a rate of injection producing a slight rise in blood pressure, the galvanometer showed a slow drift such as one observes without injection. Immediately afterwards heat was applied to one junction and after temperature equilibrium had been established, the spot of light was moved to a convenient point on the galvanometer scale. In figure 1 b, first record, the same rate of injection (0.00025 mgm. per kilo per minute, which was without effect with the heat turned off) now produced a deflection of the galvanometer in the direction of increased blood flow with a return towards the base line after the injection. One-half of the preceding rate caused a smaller increase in blood flow, while one-fourth the rate of injection (0.00006 mgm. per kilo per minute) no longer had a detectable effect on blood flow through muscles, a rate which would have been suitable for a study of the effects of epinephrine on carbohydrate metabolism.[2] In figure 1 c, a pressor rate of injection

[2] No blood sugar determinations were done in this animal, so that it is impossible to say whether or not it would have responded to this rate of injection. In other animals which were tested in a similar manner before proceeding with the blood sugar

produced a very marked increase in blood flow.[3] Incidentally, this and double this rate of injection were chosen by Soskin et al. in their experiments. Figure 1 d shows again a control procedure with the heat turned off; the injection did not produce a deflection of the galvanometer.

In the experiments in figure 2 a and b the thermocouple was placed alternately in a muscle and alongside the femoral vessels of a cat and with the same rate of injection of epinephrine an increased blood flow was observed in the former and a decreased blood flow in the latter case. In figure 2 c and d larger doses of epinephrine produced correspondingly larger effects, these being again opposite in muscle and in the whole leg. The explanation for these findings lies in the fact that a large proportion (perhaps 50 per cent) of the blood carried by the femoral vein of a resting leg comes from structures other than muscle (skin, bone marrow, etc.), so that the vasodilatation produced in muscle by injection of epinephrine is more than compensated for by the vasoconstriction occurring in the other structures of the leg, particularly the skin. This interpretation is in accord with data recorded in the literature. Thus, Gruber (8), measuring the outflow of blood from a muscle vein of a cat, noted that small doses of epinephrine caused an increased blood flow through muscle, while Hoskins, Gunning and Berry (9) showed that simultaneously with the vasodilatation in muscle, the skin vessels are constricted by injection of epinephrine.

Since blood for analysis was drawn from the femoral vein, blood flow was determined through the whole leg rather than through some individual muscle. Consequently, in the experiment in figure 3, a continuous record[4] was taken with the thermocouple placed alongside the femoral vessels and a dose of epinephrine was injected which produced little, if any, change in blood flow during a trial injection (fig. 3, a) and during the subsequent 2 hours of injection (fig. 3, b to e). Blood was drawn at the times indicated and it may be seen that no change in blood flow occurred during the sampling of the blood. At the termination of the experiment when the animal was still in excellent condition, double the rate of injection used during the experiment produced a rise in blood pressure and a decrease in blood flow through the leg corresponding to 4.5 microvolts, an indication that the blood flow recorder had not lost its sensitivity and that the epinephrine solution was still active. Finally the saphenous vein was clamped,

determinations, it was generally possible to inject epinephrine at a rate of 0.00025 mgm. per kilo per minute without producing any change in blood flow. Such rates invariably caused hyperglycemia.

[3] It should be noted that the time relations between blood pressure and blood flow are somewhat distorted owing to the fact that the lever recording blood flow writes along an arc.

[4] For the purpose of reproduction only the salient points of the record could be shown. In figure 3, b a shift in the zero point of the galvanometer occurred, the magnitude of which is indicated by the oblique line below the blood flow record.

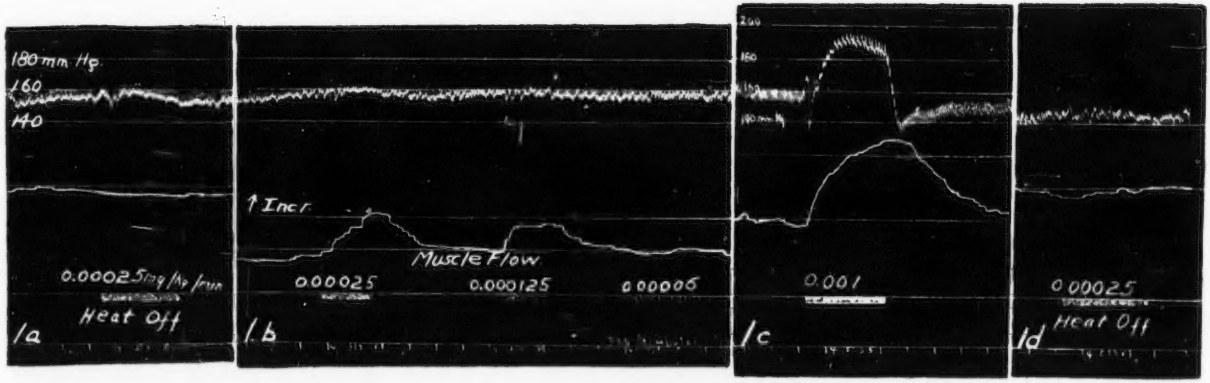

Fig. 1. Dog. Top tracing, blood pressure from carotid artery. Second tracing, blood flow record from gastrocnemius muscle. Third tracing, injection signal. Bottom tracing, time in minutes.

a and d. Control procedures. See text.

b and c. Effect of varying rates of intravenous injection of epinephrine on blood pressure and blood flow.

which produced a deflection of the galvanometer corresponding to 10.5 microvolts and blood was drawn from the saphenous vein at a rate of 6.5 cc. per minute, which produced a deflection of 1.5 microvolts. The conditions of the experiment were such that a change in blood flow of 10 to 15 per cent could have been detected; the record shows that changes of this magnitude did not occur during the injection of epinephrine.

In a number of other experiments on dogs in which a continuous record was taken, the changes in blood flow, if any, were equally small. Blood flow was not measured in the experiments on cats. Unfortunately, there is a considerable individual variation in the hemodynamic response of cats and dogs under barbiturate anesthesia to injected epinephrine; some animals were extremely sensitive, so that they could not be used in these experiments. This variability is probably due to the anesthetic rather

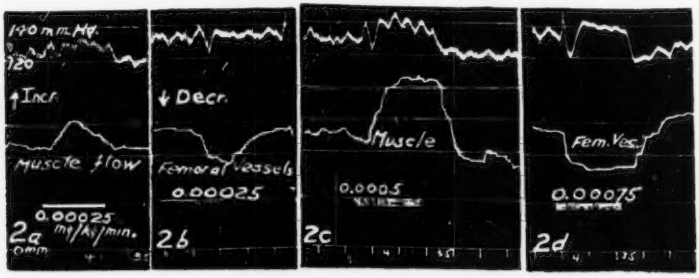

Fig. 2. Cat. Same arrangement as figure 1. Effect of intravenous injection of epinephrine on blood flow in muscle and in the whole leg.
a and c. Blood flow record from adductor muscle.
b and c. Blood flow record from femoral vessels.

than to an inherent peculiarity of the species. It was noted previously (7) that the blood pressure of rabbits anesthetized with amytal responded to smaller doses of epinephrine than that of unanesthetized rabbits.

Destruction of epinephrine in vitro. The rapid destruction of dilute epinephrine solutions, such as are used for prolonged intravenous injection, is a well-known phenomenon. It has been studied, to mention only two recent papers, by Wiltshire (10) and by Welch (11). The former author showed that at pH 7.4 most of the activity of an epinephrine solution of $1:10^6$, as measured on isolated intestinal loops, is lost in 1 minute unless the solution is protected by serum or by certain amino acids. Welch, who measured the O_2 consumption of epinephrine solutions in the Warburg apparatus, confirmed the observation of Wiltshire that amino acids protect dilute epinephrine solutions against oxidation. He also showed that sulfhydril compounds are particularly effective as anti-oxidants. We have

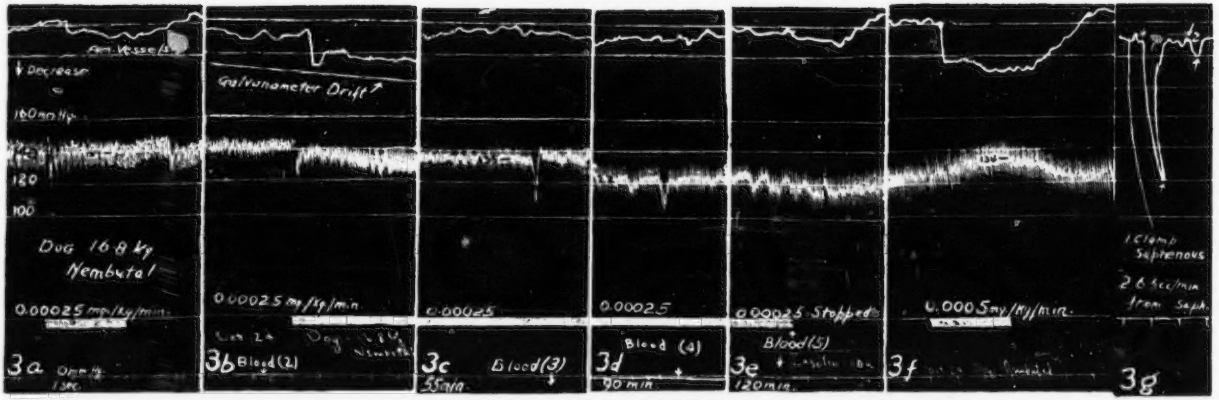

Fig. 3. Dog. Top tracing, blood flow from femoral vessels central to saphenous vein. Second tracing, blood pressure from carotid artery. Third tracing, injection signal. Bottom tracing, time in minutes. For chemical data see dog 3 in table 1.

a. Preliminary test for rate of injection to be used during experiment (0.00025 mgm. epinephrine per kgm. per min.).

b to e. Parts of continuous blood flow and blood pressure record before, during, and after injection of epinephrine. Blood samples were taken at times indicated.

f. Effect of double the rate of injection used during experiment (0.0005 mgm. epinephrine per kgm. per min.).

g. Changes in blood flow through femoral vessels produced by clamping saphenous vein and by withdrawing blood from it at the rate of 6.5 cc. per minute.

made use of this observation and have added, in recent experiments, small amounts of glutathione to our epinephrine solutions instead of serum or amino acids which were used previously.

It is a relatively simple matter to find out whether or not a destruction of epinephrine occurs during a prolonged intravenous injection. It is merely necessary to determine, at the end of the experiment, the minimal rate of injection which will raise blood pressure and then to prepare a fresh epinephrine solution and test it for its minimal pressor rate. Such tests have been carried out in a number of the experiments reported in this paper.

That such tests are necessary is illustrated by some observations made several years ago. In a device for slow injection, consisting merely of a burette, a short piece of ordinary red rubber tubing and an injection needle, the epinephrine solution reaching the animal was completely inactivated. Similarly, injections given with a Woodyatt pump caused an almost complete inactivation of the epinephrine solution. In the former case the loss of activity was caused by substances contained in the rubber tubing, because when a piece of urinary catheter tubing was substituted, no inactivation occurred. In the latter case traces of heavy metal given off by the pump were probably responsible for the destruction, since heavy metals are known to catalyze the oxidation of epinephrine. Hence, it is advisable to use glass distilled water for the preparation of the epinephrine solutions and to keep the pH at about 6. When proper precautions are used, dilute epinephrine solutions may remain fairly stable even if not protected by anti-oxidants.[5] Since so many factors enter into the inactivation of epinephrine, it is impossible to say without carrying out adequate tests, whether or not inactivation occurred with a given piece of injection apparatus. These facts are mentioned here because there are experiments reported in the literature in which dilute epinephrine solutions were injected continuously for 24 hours without protection of the solutions against oxidation and without control of the activity of the epinephrine solution at the end of the injection period.

The experiments of Soskin et al. afford an example of inactivation of epinephrine in vitro. In their experiment 54, the blood sugar values (in milligrams per cent) in the femoral vein during injection of epinephrine at a rate of 0.06 mgm. per kilo per hour were: 15 minutes, 104; 30 minutes, 105; 70 minutes, 88; 100 minutes, 76; 130 minutes, 81; 160 minutes, 72. With one-half of this rate of injection we observed a rise in blood sugar to 330 mgm. per cent (see dog 1, table 1). In two other experiments (no. 58 and fig. 3) Soskin et al. doubled the rate of injection and secured a maximal rise in blood sugar in the femoral vein of 27 and 80 mgm. per cent respec-

[5] A comparison of epinephrine solutions protected with the animals' own serum and of solutions containing glutathione as anti-oxidant has not shown a greater activity of the latter solution as regards blood sugar and blood pressure response.

tively, as compared to rises of 89, 66, 31 and 88 mgm. per cent in our experiments in table 1 with $\frac{1}{5}$ the rate of injection. The differences in the effective rates of injection of epinephrine are so large that individual variations in the hyperglycemic response of the dog cannot be held responsible. In two further experiments (figs. 2 and 4) of these authors, in which an epinephrine injection at the above high rate was superimposed upon a glucose injection, there was no apparent effect on the slope of the venous blood sugar curve. It appears then that with the exception of one or perhaps two experiments, there was practically no epinephrine activity left. We believe, therefore, that various effects which these authors ascribe to the injection of epinephrine, were due to other causes. The decline in blood flow to extremely low values in each experiment, the large and sudden changes in the hydration of blood and cases where venous blood contained up to 60 mgm. per cent more sugar than arterial blood, suggest that the methods used were not adequately controlled.

Changes in arterial and venous plasma sugar and hydration of blood. The plasma sugar[6] curves during constant intravenous injection of epinephrine in dogs and cats (tables 1 and 2) show a rapid initial rise, followed by a plateau which is maintained for 2 to 3 hours, i.e., as long as epinephrine is being administered. The height to which the plasma sugar curve rises before it levels off[7] depends on the rate of injection. Thus in dog 1, table 1, 0.03 mgm. of epinephrine per kilo per hour gave a plasma sugar level around 300 mgm. per cent, while in the other dogs receiving one-half this rate of injection the plasma sugar was maintained at 140 to 200 mgm. per cent. For an equal dose per kilo, cats show a much greater rise in plasma sugar than dogs. The sensitivity of cats approaches that of man and rabbits, 0.003 mgm. per kilo per hour being about the minimal rate at which a blood sugar response is obtained.

If the arterio-venous difference is to be used as an index of sugar utilization in the tissues, rapid changes in the glycemic level prior to the sampling of the blood have to be avoided, for the following reasons. It has been shown (12) that an increase in the plasma sugar concentration is accompanied by a corresponding increase in the sugar concentration of muscle and vice versa, so that the distribution ratio remains approximately the same at different plasma sugar levels. Hence, during a rapid rise in blood sugar there occurs a diffusion of sugar into the tissues and during a rapid fall sugar may actually be given off by the skin which contains about the same sugar concentration as plasma (Folin et al., 13). The arterio-venous differ-

[6] It seemed preferable to determine sugar in plasma rather than in whole blood because in dog and cat blood most of the sugar is present in the plasma and because the exchange of sugar takes place between plasma and tissues.

[7] In rabbits, during constant intravenous injection of epinephrine, the blood sugar curve shows much less tendency to level off than in dogs and cats.

TABLE 1

Effect of epinephrine and insulin on plasma sugar in artery and femoral vein of dogs

Dog 1 received 0.03, the others 0.015 mgm. of epinephrine per kilo per hour. A continuous blood flow and blood pressure record was taken in dogs 3, 4 and 5 (cf. fig. 3 for dog 3). All values are given in milligrams per 100 cc. A = arterial, V = venous, D = difference between venous and arterial plasma.

MINUTES	DOG 1 PLASMA SUGAR			DOG 2 PLASMA SUGAR			DOG 3 PLASMA SUGAR			DOG 4 PLASMA SUGAR			DOG 5 PLASMA SUGAR		
	A	V	D	A	V	D	A	V	D	A	V	D	A	V	D
Before injection															
60	112	111	−1	131	130	−1	143	138	−5	110	108	−2	116	115	−1
2	112	113	+1	124	122	−2	139	137	−2	109	107	−2	141	136	−5
During injection of epinephrine															
30										143	137	−6	204	203	−1
60	319	318	−1	184	185	+1	212	203	−9	141	138	−3	231	224	−7
90	331	328	−3	209	211	+2	194	189	−5	132	132	±0	230	221	−9
120	333	329	−4	202	199	−3	179	173	−6				224	222	−2
180	322	320	−2	196	192	−4									
After insulin															
20				114	89	−25	149	133	−16				167	155	−12
40				58	43	−15	95	85	−10						

TABLE 2

Effect of epinephrine and insulin on plasma sugar in artery and femoral vein of cats

Cat 1 received 0.03, cats 2 and 3 0.015 and cat 4 0.006 mgm. of epinephrine per kilo per hour. Blood pressure was recorded. All values are given in milligrams per 100 cc. A = arterial, V = venous, D = difference between venous and arterial plasma.

MINUTES	CAT 1 PLASMA SUGAR			CAT 2 PLASMA SUGAR			CAT 3 PLASMA SUGAR			CAT 4 PLASMA SUGAR		
	A	V	D	A	V	D	A	V	D	A	V	D
Before injection												
60	98	95	−3	117	114	−3	102	96	−6	186	182	−4
2	92	89	−3	139	132	−7	107	100	−7	197	191	−6
During injection of epinephrine												
60	461	451	−10	289	282	−7	440	432	−8	292	289	−3
90							437	433	−4	295	297	+2
120	465	462	−3	277	273	−4				309	304	−5
After insulin												
30				169	158	−11	293	282	−11			
60				55	47	−8	282	193	−14	230	223	−7

ence, when determined during a period of rapid fluctuation in blood sugar has therefore little meaning in terms of actual sugar utilization in the tissues, a point which has not received sufficient attention. The experiments here reported are free of this objection because most of the measurements of the arterio-venous difference during injection of epinephrine fall in a period of evenly maintained hyperglycemia.

Dog 1, table 1, received epinephrine at a rate which produced a rise in blood pressure of 10 mm. Hg. In such a case one finds a decrease in the blood flow through the leg, so that the sugar uptake is actually smaller than is indicated by the arterio-venous differences. Blood flow was not measured in dog 2, however epinephrine was given at a rate which changed blood flow only slightly in dogs 3, 4 and 5. In 5 out of 18 measurements during injection of epinephrine the arterio-venous difference exceeded the maximum difference found before the injection. On an average the differences in table 1 were 2 mgm. per 100 cc. for the two periods before injection and 3.5, 4, 3, 4 and 3 mgm. per 100 cc. for the 30, 60, 90, 120 and 180 minute intervals during the injections of epinephrine. In experiments on rabbits (14) it was found that the arterio-venous differences were much larger when the same degree of hyperglycemia was produced by injection of glucose instead of epinephrine.

The results obtained on cats (table 2) were similar to those found on dogs. In the first 3 experiments epinephrine was injected at rates which increased blood pressure and which would therefore lead to a decreased blood flow through the leg. The rate of injection in cat 4 was one-half that required to give a minimal rise in blood pressure. In some of the experiments on dogs and cats insulin was injected at the end of the epinephrine period; the decrease in plasma sugar was accompanied by an increase in the arterio-venous difference.

Experiments on rabbits (14) have shown that the tissues of the leg add an increased amount of lactic acid to the blood when epinephrine is injected. In the experiments in table 3 sugar uptake and lactic acid production were measured simultaneously (by analyzing arterial and venous blood of the leg for these constituents) and at the same time a continuous blood flow record was taken with the thermocouple inserted in the gastrocnemius. In both cases there occurred a slight increase in blood flow through muscle during the injection of epinephrine with a prompt return to the original blood flow when the injection was discontinued. These experiments show that the increased rate of lactic acid formation during epinephrine injection is not due to vasoconstriction produced by epinephrine in muscle.

Only small differences in the water content of arterial and venous plasma were encountered (see table 4). Corrections for the difference in water content were not applied, because in the majority of cases such corrections

would have changed the plasma sugar less than 1 per cent. Furthermore, it is unlikely that the exchange of fluid between blood and tissues involves water only. The dilution or concentration of blood during its passage through the tissues must be due in part to an in- and outflow of fluid containing sugar. The fact that the sugar concentration in blood plasma and lymph is of similar magnitude and shows parallel changes after insulin and epinephrine injections is in line with this point of view. We are therefore inclined to question the statement of Soskin et al. that the arterio-

TABLE 3

Comparison of the effects of epinephrine on arterio-venous blood sugar and blood lactic acid in dogs

Epinephrine was injected at a rate of 0.015 mgm. per kilo per hour. A continuous blood flow and blood pressure record was taken in both cases. Whole blood was analyzed. All values are given in milligrams per 100 cc. A = arterial, V = venous, D = difference between venous and arterial blood.

MINUTES	DOG 6						DOG 7					
	Blood sugar			Blood lactic acid			Blood sugar			Blood lactic acid		
	A	V	D	A	V	D	A	V	D	A	V	D
Before injection												
60							91	88	−3			
2	108	105	−3	22	23	+1	80	77	−3	20	21	+1
During injection of epinephrine												
20	165	156	−9	26	29	+3	96	92	−4	35	41	+6
30							100	97	−3	43	47	+4
40	180	174	−6	28	32	+4	103	101	−2	48	53	+5
60	178	170	−8	31	35	+4	107	104	−3	53	60	+7
After insulin												
20	154	143	−11				83	70	−13	40	40	±0

venous blood sugar differences are not valid unless corrected for by the differences in hydration of arterial and venous blood.

Discussion. The present experiments may be regarded as a critical examination of the value of the arterio-venous difference as a measure of sugar uptake in the tissues of the leg. The blood as drawn from the femoral vein contains not only blood from muscle, but also a large proportion of blood coming from the skin and other tissues. Furthermore, the skin and muscle vessels are influenced in an opposite direction by epinephrine; while the former are constricted by a given rate of injection, the latter are dilated, the net result being a decrease in the blood flow through the leg.

Even if changes in blood flow are avoided by suitable rates of injection of epinephrine, there remains the large error involved in the determination of the arterio-venous difference. Taking an example from the protocols, in dog 3, table 1, the first pair of plasma sugars showed 143 in the artery and 138 in the vein, a difference of 5 mgm. per cent. Assuming that under optimal conditions the error involved in the analysis of 1 cc. of plasma with the Shaffer-Somogyi 1 gram KI reagent is ± 1 mgm. per cent, then a difference of $144 - 137 = 7$ or of $142 - 139 = 3$ mgm. per cent might also have been found. With a blood flow of 40 cc. per minute through the leg, the sugar retention in the 3 cases would be 120, 168, and 72 mgm. per hour,

TABLE 4

Effect of epinephrine and insulin on water content of arterial and venous plasma

All values are given in per cent. A = arterial, V = venous plasma. The respective plasma sugar values are given in tables 1 and 2.

MINUTES	DOG 1		DOG 3*		CAT 1		CAT 2		CAT 3		CAT 4	
	H_2O Content		H_2O Content		H_2O Content		H_2O Content		H_2O Content		H_2O Content	
	A	V	A	V	A	V	A	V	A	V	A	V
Before injection												
60					91.37	91.37	92.13	92.12				
2	92.65	92.50	91.80	91.79	91.52	91.45	92.21	92.26	93.06	93.08	93.02	92.88
During injection of epinephrine												
60	92.73	92.71	91.90	91.96	92.24	92.10	92.24	92.20	92.93	92.87	92.74	92.55
120	93.40	93.30	92.22	92.22	92.05	92.20	92.24	92.31			92.96	92.92
After insulin												
30							92.10	92.09				
40			92.32	92.29								
60									92.15	92.13	92.78	92.66

* Heparin plasma.

respectively. It seems obvious that in view of this large error little can be gained by a calculation of the sugar uptake of the leg by means of blood flow figures, however accurately the latter may have been determined. The best that can be hoped for is that the anterio-venous difference may be a qualitative index of the amount of sugar taken up by the tissues. Since this method of determining sugar utilization in the tissues has found wide clinical application, it is of some importance to know whether it accomplishes its purpose.

Procedures which are known to increase the rate of glycogen deposition in muscle such as glucose or insulin injection, or a combination of both

are generally accompanied by an increase in the arterio-venous difference of the leg. During epinephrine hyperglycemia, on the other hand, the arterio-venous differences do not become so large as during a comparable degree of hyperglycemia produced by glucose injections. This has been used as an argument for the assumption that blood sugar utilization in the tissues (i.e., glycogen formation and oxidation) does not increase during epinephrine injection. If this argument were based entirely on measurements of arterio-venous differences, one might question its validity. The writers have regarded such measurements merely as a corroborative piece of information. There is actually direct evidence available that the rate of glycogen deposition in muscle and oxidation of injected glucose is decreased during epinephrine action, insulin and epinephrine being antagonists in this respect.[8]

There is still another factor which has to be considered in conjunction with the sustained rise in blood sugar during epinephrine injection. There is agreement that during the initial period of epinephrine action liver glycogen is broken down at a rate more rapid than normal and that the blood sugar rises to a new level which represents a balance between sugar production in the liver and utilization of sugar in the tissues plus kidney excretion, if any. At about this time, however, the liver glycogen has fallen to a low level, while the hyperglycemia continues as before, so that it becomes necessary to assume that the liver is forming sugar from sources other than the preëxisting glycogen stores. One of these sources has been found to be the lactic acid leaving the muscles, which is transformed to carbohydrate in the liver.

The question is whether enough lactic acid is transformed in the liver to explain the maintenance of a high blood sugar level. The transformation is certainly not rapid enough in normal animals, because injections of large amounts of lactic acid produce only a moderate hyperglycemia, but it might be of sufficient magnitude in animals injected with epinephrine in which glucose oxidation and storage of muscle glycogen occur less rapidly than normal.

Exact measurements of the transformation of lactic acid (and other metabolites) to glucose have not been reported, but the rate of glucose utilization in animals injected with epinephrine has been determined. Colwell and Bright (17) found that sugar uptake in the tissues was suppressed completely after 6 hours of intravenous injection of glucose plus epineph-

[8] Under certain conditions, for instance in rats in the post-absorptive state, epinephrine has been found to increase carbohydrate oxidation (15); a carbohydrate balance showed that this increased oxidation occurred at the expense of preexisting muscle glycogen and not of blood glucose. The increased carbohydrate oxidation observed by Dill et al. (16) in exercising dogs injected with epinephrine, is probably of the same nature.

rine, the injected sugar appearing quantitatively in the urine. The writers have confirmed the observation of Colwell and Bright, though they have pointed out that one is dealing with a combined effect of amytal anesthesia and epinephrine (18). According to Soskin et al., however, the sugar uptake in the tissues of the leg, as measured by the arterio-venous difference, is not diminished during a period of time during which Colwell and Bright found complete excretion of the injected sugar in the urine and respiratory quotients maintained at the fat-protein level. Soskin et al. assume, therefore, that the sugar excretion in the urine and the respiratory quotient bear no necessary relationship to the amount of sugar utilized in the tissues, the idea being that in addition to the injected sugar new carbohydrate is formed in the liver, presumably from fatty acids. It seems to the writers that the experiments published by these authors do not warrant these conclusions for the reasons which have been mentioned earlier in this paper.

The idea that epinephrine injection causes a transformation of fatty acids to carbohydrate has come up once before in conjunction with the effect of epinephrine on sugar excretion in diabetic dogs. Those who subscribe to this idea will be inclined to locate the hyperglycemic action of epinephrine entirely in the liver, while those who believe that such a transformation does not occur to a measurable extent in the mammalian organism, deny the possibility that overproduction alone can explain the maintenance of a high blood sugar level during epinephrine injection. Though the experiments reported in this paper are in line with those of Colwell and Bright, they do not settle the problem, because it has so far not been possible to inhibit glucose utilization by means of epinephrine injections in liverless animals.

SUMMARY

1. It has been shown that it is important to protect dilute epinephrine solutions against destruction in vitro by addition of anti-oxidants. With such protected epinephrine solutions a rate of intravenous injection of 0.015 and of 0.003 mgm. per kilo per hour produced hyperglycemia in amytalized dogs and cats, respectively.

2. The use of a blood flow recorder made it possible to find rates of injection of epinephrine which increased plasma sugar and lactic acid without changing significantly the blood flow through muscle or through the whole leg. Rates of injection somewhat larger than those required to increase blood sugar caused an increased blood flow through muscle and a decreased blood flow through the whole leg, this being attributed to the fact that vasoconstriction of skin vessels more than compensates for the vasodilatation occurring in muscle.

3. The arterio-venous plasma sugar difference was determined in dogs

during a period of evenly maintained hyperglycemia, epinephrine being injected at a constant rate. The average difference which was 2 mgm. per cent before the injection, varied between 3 and 4 mgm. per cent during the injection of epinephrine. Insulin injected at the termination of the epinephrine period resulted in differences of 10 to 25 mgm. per cent. Similar results were obtained on amytalized cats, except that this species responded, for an equal dose per kilo, with a much greater rise in plasma sugar than did dogs. It was not found necessary to correct the plasma sugar values for changes in the hydration of the blood during its passage through the tissues of the leg.

4. The significance of the arterio-venous difference as a measure of sugar utilization in the tissues and the errors involved in such determinations have been discussed, as well as the current theories of the mechanism of epinephrine hyperglycemia.

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AFFERENT FUNCTION IN THE GROUP OF NERVE FIBERS OF SLOWEST CONDUCTION VELOCITY[1]

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Afferent function in the unmyelinated fibers of the dorsal roots was suggested many years ago by the work of Ranson and Billingsley (15, 16, 18) and has been the subject of more recent investigation by Ranson and Davenport (17, 19). The experiments of Erlanger and Gasser (6), Adrian and his co-workers (1, 2, 3, 4), Tower (20), and Hogg (13) have also given indications that nerve fibers of small size and slow conduction velocity convey afferent impulses. Up to the present time, however, no demonstration has been made of reflex effects produced by stimulation of afferent nerve fibers definitely shown to be of the group with slowest conduction velocity, the "C" fibers.

METHODS AND RESULTS. The possibility that fibers of the "C" group may have an afferent function has been investigated by the observation of reflex effects in the cat obtained under the following conditions:

1, the effect of electrical stimulation of the saphenous nerve at strengths sufficient to excite only the "A" and "B" fibers, in comparison with the effect of stimulation of the "C" fibers as well as the "A" and "B" groups;

2, the effect of electrical stimulation of the "C" fibers after all the "A" and "B" fibers had been blocked by compression or asphyxia;

3, the effect of mechanical stimulation of the "C" fibers after the "A" and "B" fibers had been blocked.

The saphenous nerve was selected for stimulation because its anatomical composition has been thoroughly investigated by Ranson and Davenport (19) and by Heinbecker, O'Leary, and Bishop (12), who showed that after sympathectomy and ventral root section large numbers of unmyelinated fibers of dorsal root origin are still present in this nerve. The Harvard inductorium with variable resistance in the primary circuit was used throughout for stimulation. Reflex effects were judged by changes in the rate and amplitude of the respirations and, in a few experiments, changes in the blood pressure.

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Cats, for the most part under light dial or amytal anesthesia, were used throughout. It is known from the work of Heinbecker, Bishop, and O'Leary (10, 11) that stimulation under ether at a strength sufficient to excite the fibers of the "A" and "B" groups produces marked reflex effects. In their experiments no additional effects upon the animal could be demonstrated when the "C" group was also stimulated. Under barbiturate anesthesia, however, the effects of stimulating the "A" and "B" fibers alone are re-

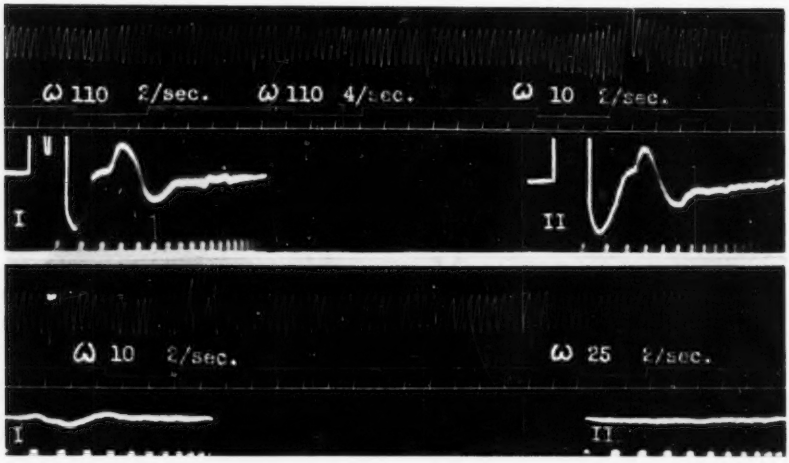

Fig. 1 (above). Pneumograph tracing and electroneurogram during stimulation of cat's saphenous nerve. Dial anesthesia. 110 ohms, etc., = resistance in primary circuit of induction coil. 110 ohms = "B" strength; 10 ohms = "C" strength. Rates shown are the number of make shocks and break shocks per second; thus 2/sec. means 2 makes and 2 breaks per second. Time for pneumograph tracing in 6 second intervals. *I* is the electroneurogram at stimulus strength, 110 ohms, showing shock artifact, lower part of "A" elevation, and full "B" elevation. *II* is the electroneurogram at 10 ohms. Time for the electroneurograms in 1 millisecond intervals.

Fig. 2 (below). Later section from same experiment as figure 1. *I* is electroneurogram at 10 ohms stimulus strength, showing "C" elevation. *II* is at 25 ohms, "C" elevation no longer present. Time for the electroneurograms is in 16.6 millisecond intervals.

duced to a minimum, while striking changes in respiration still accompany stimulation at "C" strength. Such differential effects are illustrated in figures 1 and 2. It will be seen in figure 1 that but slight effects upon respiration occur with "B" stimulation (110 ohms) and that these effects are of similar degree at rates of either 2 per second or 4 per second. Stimulation at "C" strength (10 ohms), 2 per second, however, produces a considerable increase in both rate and depth of respiration. The action

potentials (*I* and *II*) shown below the pneumograph tracing were recorded during the corresponding periods of stimulation. The shock artifact, lower part of the "A" elevation, and the full "B" elevation can be seen in them, but the "C" elevation is not visible because the oscillograph sweep is too fast. With a slower sweep, however, "C" could be seen on the oscillograph at 10 ohms (see fig. 2). There is a slight deficit in the area of the "B" elevation in figure 1, *II*, when the "C" strength stimulus is applied. This deficit is probably due to the fact that a few "B" fibers were stimulated at the lead electrodes rather than at the true cathode. The area of their potential is therefore masked in the large shock artifact. The deficit itself, however, shows that these fibers did not discharge repetitively, for if they had done so the area of their potential would also have been repeated. Examination of the electroneurogram at lower amplification in a number of experiments showed that any area of "B" which appeared under the shock artifact was then absent from its usual position in the electroneurogram. Any experiment in which such an area was seen to repeat itself was excluded from the series. Observations upon the "A" fibers in several experiments showed similar characteristics with respect to repetitive discharge, and such a possibility was controlled in the same manner as with the "B" fibers. When repetitive discharge is thus controlled, the clear inference is that the increased reflex effects obtained at "C" strength were due to a true afferent function of "C" fibers and not to repeated responses in the "B" fibers. This inference is further supported by a number of experiments similar to that shown in figure 1 in which "B" strength stimulation at rates two or more times as rapid as those employed at "C" strength still failed to produce reflex effects as marked as those found when the "C" fibers were also stimulated, although at lower rates.

Figure 2, taken from the same experiment as figure 1, illustrates another method of controlling the possibility of repetitive discharge from the "A" or "B" fibers. It will be seen in the first instance (*I*) that "C" strength shocks (10 ohms) produce definite respiratory effects and a "C" elevation in the electroneurogram. In this figure the action potential records are made from a slow oscillograph sweep; the "A" and "B" elevations are not shown in *I*, although they could be seen with a faster sweep. Shocks but slightly below "C" strength (25 ohms, *II*) however, fail to produce much reflex effect and the "C" elevation is absent. This and a number of similar experiments show that it is, in fact, precisely at the moment of the appearance of the "C" elevation that the increase in reflex effects takes place. Gradual increase in the shock strengths after the "B" elevation had reached its maximum size consistently failed to produce any changes in reflex effects until the "C" elevation appeared in the electroneurogram—at which point the greater effects invariably occurred. There is nothing so crucial as this in the occurrence of repetitive discharge in the "A" and "B" fibers.

This, when it occurs, may appear at any point above maximal stimulation of these fibers, regardless of whether or not the "C" elevation is present.

Such evidence of additional afferent impulses entering the nervous system during stimulation of the "C" fiber group was found without exception in numerous trials upon 21 animals. (One dog was used in this series.)

The second method employed was the stimulation of the "C" fibers after the elimination of the "A" and "B" groups by means of the pressure chamber introduced by Gasser and Erlanger (8, 9). This chamber consists of a brass cuff of small bore which contains a soft rubber tube. The nerve was dissected out, cut away at the ankle, and the free end threaded through the rubber tube, while the proximal portion remained in full connection with the animal. Stimulating electrodes were placed upon the nerve distal to the chamber and lead electrodes proximal to it. Compres-

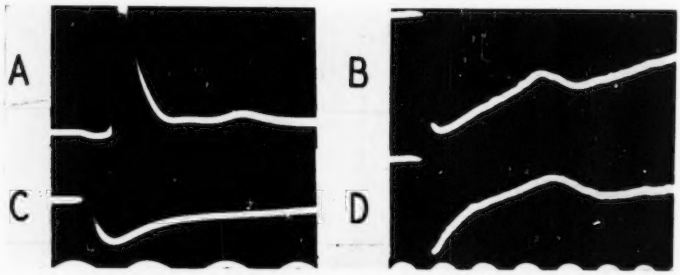

Fig. 3. Effect of compression on electroneurogram of excised cat's saphenous: *a*, "A" and "B" elevations before compression; *b*, "C" elevation before compression; *c*, "A" and "B" elevations completely eliminated by compression ("C" strength shock); *d*, "C" elevation at time when "A" and "B" have been eliminated; "C" is slightly delayed. Time for *a* and *c* in 1 millisecond intervals; time for *b* and *d* in 12 millisecond intervals.

sion was applied by the introduction of oxygen under pressure between the brass cuff and the rubber tube. After compression by this means for periods varying from 15 to 45 minutes (depending upon the amount of pressure used) only the "C" fibers remained active in the blocked region. The effect upon the saphenous nerve is shown in figure 3 which is taken from an experiment in which the nerve had been completely excised; *a* and *b* illustrate the normal electroneurogram of the cat's saphenous; *c* is taken after compression of the nerve and shows the application of a stimulus of "C" strength. There are no elevations in the "A" or "B" positions. *d*, taken immediately after *c*, shows that a good "C" elevation is still present, though somewhat late in time because the conduction of the impulse is delayed in the compressed portion of the nerve.

When this method of blocking nerve fibers is used on the saphenous of

an anesthetized cat, reflex effects may be studied before and after the application of compression. Such an experiment is illustrated in figure 4. In *I*, made before compression, a distinctly greater effect is seen when the

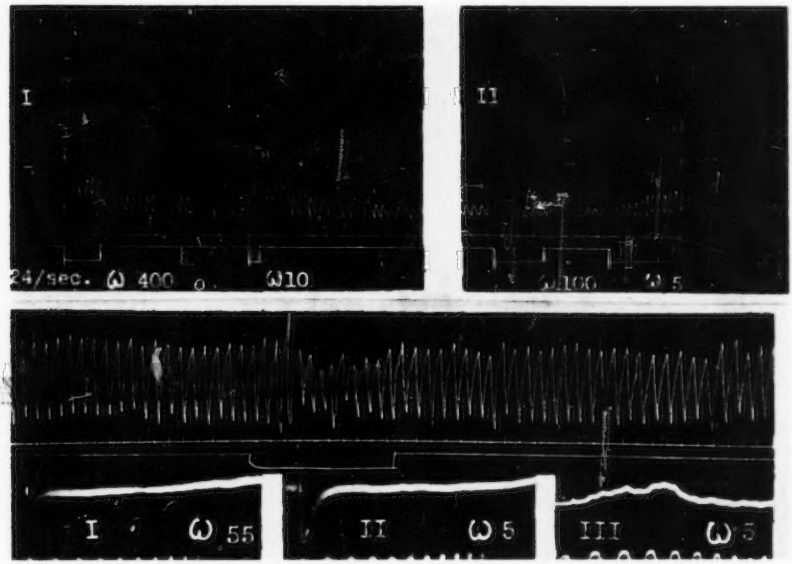

Fig. 4 (above). Blood pressure (upper line) and pneumograph tracing during stimulation of cat's saphenous. Dial anesthesia. *I*, before compression of nerve with pressure chamber. 400 ohms = "B" strength; 10 ohms = "C" strength. "A," "B," and "C" elevations were all present in electroneurogram. *II*, after compression of nerve. "C" elevation alone was present in electroneurogram. 100 ohms = "B" strength; 5 ohms = "C" strength.

Fig. 5 (below). Pneumograph tracing and electroneurogram during stimulation of cat's saphenous nerve. Amytal anesthesia. A block has been produced by asphyxia. Nerve pinched with artery forceps distal to block during time signalled. Time for pneumograph tracing in 6 second intervals. All the oscillograph records seen below the pneumograph tracing were recorded proximal to the block just before the nerve was pinched. Stimuli for these were single induction coil break shocks applied distal to the block. They do not affect the pneumograph tracing. *I*, no trace of "A" or "B" elevations present at "B" strength (55 ohms); *II*, no trace of "A" or "B" elevations present at "C" strength (5 ohms). Time for these two in 1 millisecond intervals. *III*, "C" elevation which is present at 5 ohms (much slower sweep). Time in 16.6 millisecond intervals.

"A," "B," and "C" fibers all are active (10 ohms) than when only the "A" and "B" groups are stimulated (400 ohms). It will be noticed that in this instance the "C" stimulus, although producing the greater effect, was of

much shorter duration than the "B" stimulus. In *II*, after the block is established, at which time only the "C" elevation remained in the electroneurogram, stimulation at "C" strength still causes effects upon blood pressure and respiration as great as those obtained before compression of the nerve, while no effects whatever occur upon "B" stimulation.

A nerve fiber block of very similar characteristics to that obtained with compression can also be obtained with asphyxia produced by means of a sphygmomanometer cuff surrounding the entire leg of the animal, including the saphenous nerve, and inflated above systolic blood pressure for about 45 minutes, or produced by stoppage of the circulation alone for a similar length of time (5), in which instance the nerve is not included in the tourniquet. Such a block has the advantage that it is reversible, if not too prolonged, so that it can be repeated several times on the same nerve, while the block produced by compression is irreversible. Stimulation of the saphenous distal to an asphyxial block when only the "C" elevation remained in the electroneurogram recorded from a region proximal to the block, yielded results in every way similar to those obtained after a block by compression such as that shown in figure 4. To insure against the possibility that the strong shocks needed to excite the "C" fibers might be producing their reflex effects by spreading above the block, a mechanical stimulus which by its nature cannot spread—pinching of the nerve distal to the block with artery forceps—was employed alone in several experiments. A typical result of this procedure is shown in figure 5. It will be seen that at a time when no visible elevations were present in either the "A" or the "B" positions in the electroneurogram recorded proximal to the block, but while the "C" elevation was still clearly present, pinching of the nerve resulted in respiratory changes—a reflex which on its sensory side must have been mediated by fibers of the "C" group, since they alone were active through the blocked region at this time.

Altogether, reflex effects produced by stimulation of the "C" fibers alone, after the "A" and "B" fibers had been blocked by compression or asphyxia, were obtained in repeated trials upon 17 cats. If either compression or asphyxia were prolonged sufficiently (much longer than one hour) the "C" fibers were also blocked and no further reflexes could be obtained with even the strongest stimulus. Failure to elicit reflex activity was also encountered if the anesthesia was too deep. With these exceptions, results were consistently positive.

An experiment analogous to the asphyxial blocking just described in cats has been demonstrated in man by Lewis, Pickering, and Rothschild (14) and repeated in this laboratory with entirely confirmatory results (7). In the human experiment the sphygmomanometer cuff is inflated about the upper arm and kept continuously at a point above systolic blood pressure. This causes a progressive loss of sensation distal to the cuff which takes

place in a constant order. The time relations of this loss of sensation in man furnish a striking parallel to the time relations of the changes in the electroneurogram of the cat's saphenous under analogous conditions. For the first 15 minutes, in the human experiment, no loss of sensation occurs. During a similar period the electroneurogram of the cat's saphenous shows no change except a slight slowing of conduction rates. In man, the sensations of touch, pressure, vibration, and position begin to disappear rapidly after about 15 to 20 minutes, and the sensation of cold begins to go at about the same time or slightly later. In the cat, there is rapid disappearance of the "A" and "B" elevations during this period. After 30 to 45 minutes the human subject has completely lost the sensations named above and the only responses obtainable from the area distal to the cuff by any sort or intensity of stimulus are delayed sensations of warmth and of a severe burning pain. In the cat's saphenous at this time all "A" and "B" fiber activity has been lost and the "C" fibers alone are conducting impulses through the asphyxiated region. After release of the cuff the sensations in man and the electroneurogram in the cat return in a few minutes to the normal state. Five human experiments and 15 analogous animal experiments were carried out in this series, the time relations agreeing closely in every instance with those cited above. From these observations it is inferred that the human subject, after 45 minutes of an asphyxial block, feels warmth and pain from points distal to the block as a result of afferent impulses conducted solely by "C" fibers, just as in the cat during such a block reflex effects are obtained when only the "C" elevation is present in the electroneurogram.

SUMMARY

1. When the fibers of the "A," "B," and "C" groups in the cat's saphenous nerve are all stimulated, greater effects upon respiration and blood pressure occur than when only the "A" and "B" groups are active.

2. When all the "A" and "B" fibers of the saphenous have been blocked by compression or asphyxia and only the "C" fibers remain active in the blocked region, stimulation of these fibers alone by electrical or mechanical means continues to produce reflexes.

3. We therefore conclude that "C" fibers carry afferent impulses capable of producing reflex effects in cats.

4. A close parallel in time relations exists between the disappearance of sensations in man during asphyxial nerve block and the disappearance of the elevations in the electroneurogram of the cat's saphenous nerve during a similar block.

5. After 45 minutes of asphyxial block in the cat, "C" fibers alone are conducting impulses through the blocked region; at this time in the human subject sensations of pain and warmth alone can be evoked distal to such a block.

6. It is therefore inferred that in man afferent impulses which are interpreted as pain and warmth are conducted at least in part by "C" fibers.

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THE DISCHARGE OF IMPULSES FROM PACINIAN CORPUSCLES IN THE MESENTERY AND ITS RELATION TO VASCULAR CHANGES[1]

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It has frequently been suggested that there may be vascular zones other than the arch of the aorta and the carotid sinus which play an afferent rôle in cardio-vascular reflexes. Because a considerable measure of circulatory control is achieved by variations in the volume of the vascular bed in the splanchnic area and because stimulation of the central ends of the splanchnic nerves profoundly modifies blood pressure, it seemed desirable to search in that region for receptors which are functionally related to the circulatory system.

The problem was approached by recording the impulses in the peripheral ends of the splanchnic nerves and their branches. In practically every one of a long series of experiments we found a grouped discharge of impulses synchronous with the systolic rise in pressure and often continuing in some measure throughout the cardiac cycle. The determination of the end organs which give rise to these impulses and their relation to vascular changes was the object of the work here reported.

METHODS. Several hundred experiments were made on about fifty cats. The animals were generally anesthetized with urethane or nembutal. In a few cases they were decerebrated after preliminary ether anesthesia. The splanchnic nerves and their branches were reached in the abdomen, or in the thorax just above the diaphragm with the abdomen unopened. They were cut centrally and freed from the surrounding tissues for some distance so that they could be placed on electrodes leading to a capacity-coupled amplifier and Matthews' oscillograph. In many experiments the nerve was reduced by dissection to one or a few active fibers.

RESULTS. *The end organs responsible for the discharge.* In considering what sense organs might give rise to these impulses we were impressed by the close relation of many Pacinian corpuscles to the vessels of the

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region. They lie in great abundance along the arteries, in the crotches of arterial branches in the root of the mesentery, and along the arcade arteries supplying the intestine. See figure 1. We were also reminded that the corpuscles have an intrinsic circulation.

Definite information concerning the effective stimulus for Pacinian corpuscles in another area of the body has been reported by Adrian and Umrath (1) who studied the activity of those located under the flexor tendons of the cat's toe and on the surface of the tendon sheaths. They found that the corpuscles were readily excited by pressure or mechanical deformation. We therefore decided that here in the mesentery where they

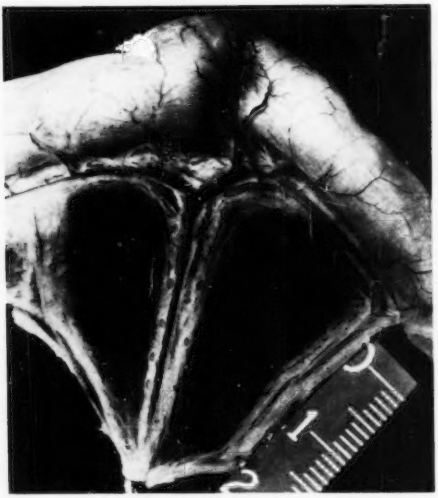

Fig. 1. Pacinian corpuscles in the mesentery of a cat.

frequently adjoin the arteries they might be stimulated by the arterial thrust during systole, or by a steady distention of the vessels, or indeed by the pressure within their own intrinsic vessels.

Final identification of the Pacinian corpuscles as the source of these impulses was made as follows. The pulsatile discharge was recorded in a small nerve twig from a few receptors. The nerve supply to one Pacinian after another was then cut with a consequent progressive diminution and final abolition of the impulse discharge as the successive corpuscles were eliminated. It can therefore be stated that the Pacinian corpuscles in the mesentery may be stimulated by vascular changes and thus give rise to the impulses which we have just described. We have not entirely eliminated the possibility that other types of endings in this region also discharge

impulses synchronously with the pulse but we have not been able to find any evidence of such activity.

In the discharge from a large group of receptors we usually find some which function only during systole and others which are active throughout the cardiac cycle. When the nerve is reduced to a few fibers one may observe only the former type (as in fig. 2) or the latter type (figs. 3, 4, and 5). The difference in the character of their discharge may be due to differences in their mode of stimulation. This will be discussed later.

Relation of the impulse discharge to perfusion pressure. In order to determine the effect of variations of intravascular pressure on the end organ response we have perfused at constant pressure a portion of the superior mesenteric arterial system with Ringer's solution or defibrinated blood. Under such conditions there is a maintained discharge of nerve impulses from the Pacinian corpuscles provided the perfusion pressure exceeds a

Fig. 2. Discharge from several Pacinian corpuscles synchronous with pulse. Time $\frac{1}{5}$ second.

certain critical level which varies considerably from preparation to preparation.

If the perfusion pressure is raised and lowered in a pulsatile manner, there is a vigorous burst of impulses accompanying each rise in pressure. The pressure necessary to set up such momentary activity is less than that required to elicit a continued discharge. This fact is in agreement with the known properties of certain other types of sense organs as well as of the Pacinian corpuscles in the joints investigated by Adrian and Umrath.

The corpuscles in these perfusion preparations are little affected by changes in body temperature and the degree of oxygenation of the perfusion fluid. Neither of these factors seems to alter the character of the discharge significantly.

Having thus found in perfusion experiments that an elevation of intravascular pressure is an effective stimulus for the Pacinian corpuscles we turned next to a study of the relation of their activity to circulatory changes in the animal.

Effects of changes in blood volume. The intravenous injection of a considerable quantity of Ringer's fluid (*ca* 200 cc.) in the intact animal causes a very great increase in the number and frequency of afferent impulses. This persists for some minutes following the injection. On the

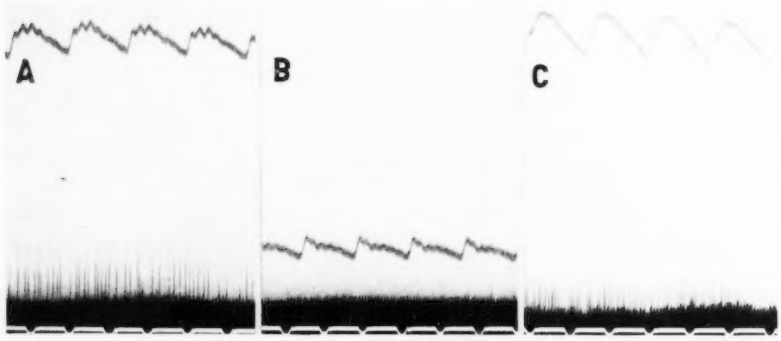

Fig. 3. The effect of hemorrhage on Pacinian corpuscle discharge. A. Control. B. After bleeding. C. After reinjection of equal quantity of blood. Time $\frac{1}{5}$ second

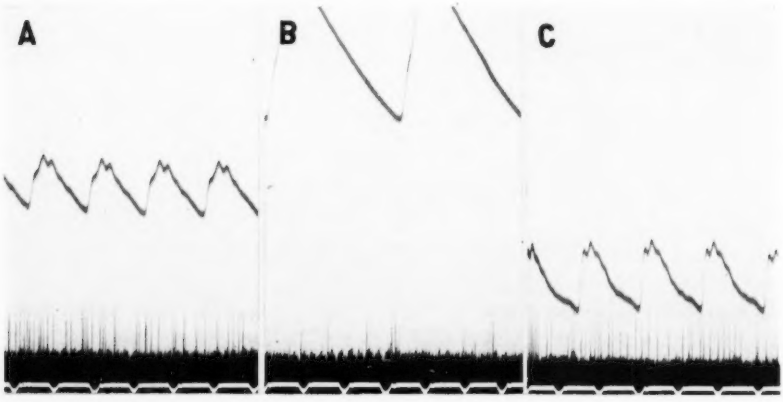

Fig. 4. The effect of vasoconstriction due to adrenalin on Pacinian discharge. A. After injection of blood. B. After adrenalin. C. After adrenalin effect had worn off. Time $\frac{1}{5}$ second.

other hand, if 50 cc. or more of blood are withdrawn from the animal, there is a prompt decrease or cessation of the sensory activity. On reinjection of an equal quantity of defibrinated blood the discharge of impulses is again resumed. Such an experiment is illustrated in figure 3. We may conclude

that one of the functions of the Pacinian corpuscles in the mesentery is to signal the degree of distention of the mesenteric vessels.

Relation of the discharge to mean blood pressure. Such observations also suggest a definite correlation between mean systemic blood pressure and the activity of the Pacinians. Injection of fluid produces a rise in blood pressure and an increased discharge from the sense organs; with hemorrhage there is a drop in pressure and decreased sensory activity. When however the variations in blood pressure were produced by certain peripherally acting drugs we failed to find such a parallelism. An instance is illustrated in figure 4. The blood pressure had been raised by an intravenous injection of defibrinated blood which produced the usual increase in sensory response. But when the pressure was further increased by an injection of adrenalin there was a marked decrease of the impulse discharge. On the

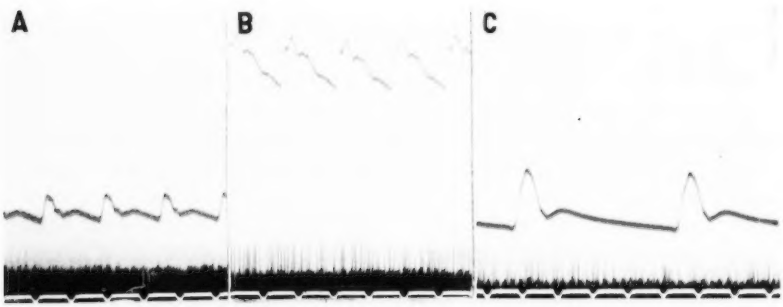

Fig. 5. Comparison of the effect of hemorrhage with the effect of vasodilatation due to acetyl-B-methylcholine. A. After hemorrhage. B. After reinjection of equal quantity of blood. C. After acetyl-B-methylcholine. Time $\frac{1}{5}$ second.

other hand (fig. 5) there was usually an increase, or at least no reduction, in the number of afferent impulses when the blood pressure was lowered by means of acetylcholine, although there was invariably a marked decrease with a similar drop in pressure resulting from hemorrhage. In general, a rise in blood pressure produced by a vasoconstricting substance causes a decrease in the end organ response; a rise in blood pressure resulting from an increased blood volume increases the number of afferent impulses. It would therefore appear that the total sensory discharge is not directly related to the level of general blood pressure but rather to the degree of distention of the mesenteric vessels as determined by the blood volume.

A discrepancy is here apparent between the behavior of the Pacinian corpuscles and that of the carotid sinus and aortic endings. Why should the Pacinians respond in an opposite manner to an increase of blood pressure caused by vasoconstriction and one due to augmented blood volume,

while the carotid sinus endings respond in a constant fashion to elevation of blood pressure regardless of the cause of that elevation? (Bronk and Stella, 2.)

The carotid sinus and aortic endings lie within the adventitia of large vessels whose degree of distention is determined by the blood pressure. The Pacinian corpuscles on the other hand have a fairly rich vascular supply of their own, as has been emphasized by Sheehan (3). This consists of a central vessel piercing the core and a network of capillaries surrounding the outside of the ending. It is at least possible that the pressure within these intrinsic vessels is in part responsible for the excitation of the sense organ. If that be so, the injection of adrenalin would cause a constriction of the arterioles with a consequent decreased distention of the vessels in the corpuscles. There would accordingly be a decreased stimulus in spite of the rise in general blood pressure. The injection of acetylcholine or amyl nitrite would dilate the small vessels and give an increased stimulation. Because such effects are generally observed they lend some support to the theory that the Pacinian corpuscles are stimulated by the pressure exerted by vessels of their intrinsic circulation. The fluctuations of pressure within these vessels would be relatively small. This type of vascular stimulus could therefore account for the less pulsatile discharge from some of the corpuscles.

It is also probable that the end organs are stimulated by the distention of the arteries along which many of them lie. It must be borne in mind that these vessels are smaller than those to which the carotid sinus and aortic endings are related and it is not unreasonable to suppose that they would be constricted by adrenalin. Under these conditions the Pacinian corpuscles would be stimulated less even though the general level of blood pressure was higher.

The relative importance of the stimulus from the intrinsic circulation of the corpuscles and that from the larger vessels which so many of these end organs adjoin can not be decided from our experiments. Nor does it appear to be possible to answer this question finally and conclusively until a technique is developed for isolating and perfusing the circulation of a Pacinian corpuscle. At the present time, however, we can say that the activity of these sense organs is definitely related to the vascular system and that they signal the degree of distention of the mesenteric vessels. It is therefore of interest to consider the possible reflex results of the afferent impulses which they discharge.

Reflex effects. We first investigated their effect on general blood pressure. This was done by perfusing the superior mesenteric artery which was isolated from the general circulation, and with the venous outflow cut off from the vena cava. No significant changes in the carotid or femoral blood pressure were observed when the perfusion pressure was varied over a wide

range. Similar results were obtained after the stabilizing influence of the aortic and carotid sinus nerves had been removed by cutting them.

Another approach was designed to test the hypothesis that these afferent impulses which result from a distention of the splanchnic vessels reflexly induce a constriction of those same vessels. The superior mesenteric artery was divided into two portions, each supplying approximately half of the small intestine. By clamping the vessels it was possible temporarily to cut off this whole area from the general circulation while the nerve supply of both portions was kept intact. The upper portion was perfused at various pressures. The lower half was perfused at constant pressure and the flow was recorded by means of a suitable flow meter which consisted of a differential manometer across a capillary. A decreased flow therefore indicated a vasoconstriction. In many experiments there was evidence of a reflex constriction of the vessels in the lower half of the mesentery when the vessels in the upper half were distended. But the results were frequently variable and must therefore be considered as suggestive rather than conclusive.

Conclusion. Although their reflex effects remain to be demonstrated the possible importance of these afferent impulses will be apparent. The splanchnic vessels play such an important rôle in the control of the circulation in normal and pathological conditions that it would have been surprising had we not found some afferent mechanism for reporting to the centers the state of the mesenteric vasculature. It is gratifying to find that the receptors responsible for these sensory messages turn out to be the Pacinian corpuscles which had often been suspected, on anatomical grounds, of having a functional relation to the circulatory system.

SUMMARY

Afferent impulses initiated by vascular distention have been observed in the peripheral ends of the splanchnic and mesenteric nerves.

These impulses are discharged from the Pacinian corpuscles in the mesentery, many of which lie in close relation to the mesenteric vessels and in addition have an intrinsic circulation of their own.

The corpuscles may be stimulated by perfusing the mesenteric circulation at pressures above a certain critical level which varies considerably from preparation to preparation. Thus elevation of the intravascular pressure either within the intrinsic vessels of the corpuscle or within the larger vessels which the corpuscles adjoin, or in both is capable of evoking the discharge.

Distention of the mesenteric vessels in the intact animal by injection of blood increases the number and frequency of afferent impulses; a decrease in the calibre of the vessels following hemorrhage causes a decrease in the afferent discharge. On the other hand, changes of blood pressure due to

peripherally acting drugs usually affect the discharge in an opposite direction from those produced by injection or withdrawal of blood. These observations suggest that the effective stimulus is related to the degree of distention of the mesenteric vessels rather than to the level of mean blood pressure. In this respect the discharge from these receptors differs from that originating in the carotid sinus and aortic end organs. It is suggested that this may be due to the nature of the intrinsic circulation of the Pacinian corpuscles.

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CHANGES IN ELECTRICAL RESISTANCE OF NERVE DURING BLOCK BY COLD AND BY HEAT

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Bahrmann (1932) reported that when frog nerves are locally cooled, the failure of impulse conduction is either accompanied or immediately followed by a sharp rise of electrical resistance, in a typical instance from 98,000 to 344,000 ohms. An increase of this order was invariably associated with block, so that some necessary connection between the two phenomena might be inferred.

The cold block in normal frog nerve is usually due to ice formation, and the low temperature limit of conduction is simply the minimum temperature to which the nerve can be supercooled. Under favorable conditions this may be as low as $-10.0°$C., although ice formation (and block) can at will be induced at -1.0 to $-2.0°$ (Boyd and Ets, 1934). Bahrmann's experimental conditions were such that nerves froze only after supercooling to an average temperature of $-6.0°$. The resistance, in a liquid conductor, would be expected to increase with ice formation, and in a supercooled system the change would naturally be rapid because of the rate at which crystallization spreads. It seemed to us worth while, therefore, to repeat Bahrmann's experiments, modifying the technique so as to minimize supercooling. We have also followed the resistance changes in nerves blocked by cold without ice formation. Such a "non-freezing" cold block can nearly always be produced in mammalian nerves, and occasionally in normal frog nerves, particularly in summer.

Our experiments were carried out during the months from January to May, inclusive, on sciatic-gastrocnemius preparations from *Rana pipiens*, *R. catesbiana*, and the albino rat. Excised mammalian muscles usually cease after a short time to respond to indirect stimulation, but Dr. H. N. Ets (unpublished) has found that the rat preparation remains in good condition for one to two hours at room temperature if soaked in a modified Locke's solution (NaCl 0.92, KCl 0.042, $CaCl_2$ 0.018 per cent). The blocking temperature of the nerve, during this time, remains constant or becomes progressively lower.

Alternating current for the resistance measurements was supplied by an

audio-frequency oscillator consisting of a triode (201-A) with feed-back through an audio-frequency transformer having a range of 1000 to 2000 cycles per second. Measurements were made by means of a conventional bridge, the resistance of the nerve being balanced by a series of non-inductive resistances with which it was possible to make readings from 1000 to 2,000,000 ohms. The other two arms of the bridge were fixed resistances of 100,000 ohms each. A variable condenser of 0.0005 mfd. maximum capacity was placed in parallel with the variable resistance. Using ear phones as an indicating device, the null point was quite satisfactory. The operating current was not strong enough to stimulate the nerve, and was usually left running throughout an experiment, with occasional changes of frequency to relieve auditory fatigue.

The arrangement of stimulating and measuring electrodes was similar to that of Bahrmann (loc. cit.). The test stimulus was a single break induced shock just above the strength necessary for a maximal muscle twitch. Bahrmann and also Bühler (1905) describe a rising threshold just previous to complete block. We considered the nerve blocked when response to the original stimulus failed, believing that stronger stimuli were effective only through current spread.

Between the measuring electrodes, the nerve lay in a transverse groove on the outer surface of a glass tube. Cooling was accomplished by allowing cold salt solution to flow at varying rates through this tube from a reservoir. When supercooling was desired, the nerve was covered with vaseline and the adjacent glass surface with rubber tubing. When it was to be avoided, nerve and cooling tube were left exposed to the air of the moist chamber. A thin film of water condensed on the glass surface as it cooled, and the gradient of temperature was such that freezing of this film always began near the reservoir before the nerve was cooled below 0° (Boyd and Ets, loc. cit). Water condensation around the nerve introduced a certain error into the resistance measurements, but considering the thinness of the film and its low conductivity the error was small. Bullfrog nerves, because of their relatively large diameter, were cooled by a different method, the nerve being drawn through a thin-walled glass tube sealed transversely through the cooling tube. Temperature was measured by means of a thermocouple in contact with the middle of the cooled segment of nerve, which usually included the entire length between the measuring electrodes. For bullfrog nerves, this length was 31 mm.; pipiens, 14, and rat nerves 11 mm.

With the cooling device used for bullfrog nerves supercooling could not conveniently be avoided. Freshly dissected preparations invariably conducted until ice formed (indicated by sudden heat liberation, causing a movement of the galvanometer in the thermocouple circuit). Freezing temperatures varied from -3.0 to $-7.0°$, and the resistance changes were

quite similar to those described by Bahrmann. On further cooling, as far as −15.0°, the resistance continued to rise rapidly. The magnitude of the increase associated with block, however, varied roughly according to the temperature at which ice was formed. Contrary to the observations of Bahrmann, the capacity in the balancing circuit had to be reduced, rather than increased, as the nerve froze.

Pipiens nerves subjected to freezing block at −1.0° to −2.0° behaved somewhat differently. The resistance curve shows a definite break, but

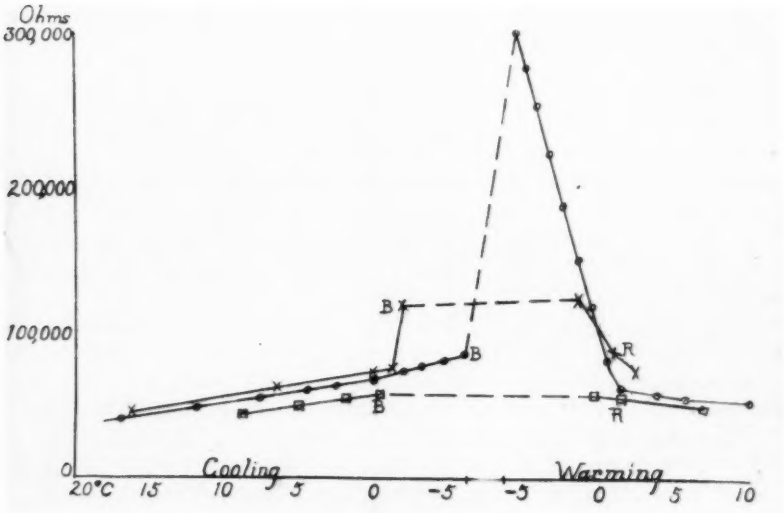

Fig. 1. Resistance changes associated with cold block. ○, bullfrog nerve, 31 mm. segment, freezing block after subcooling to −5.9°C. ×, pipiens nerve, 14 mm. segment, freezing block at −1.5°. □, rat nerve, 11 mm. segment (R multiplied by 3 in order to bring it into scale), block without freezing at −0.5°. B block, R recovery (none after freezing at −5.9°).

the maximum reached is less than double the initial value, as compared to the three- to five-fold increase seen with freezing at −6.0°. The onset of block is also gradual, a weakened muscle response usually persisting for some time after freezing begins (fig. 1). In the supercooling experiments, the break in the resistance curve indicates accurately the point of complete block; at −1.5° it shows only the onset of partial block.

The non-freezing cold block is not associated with any break in the resistance curve. This we have verified on normal rat nerve (fig. 1), on frog nerves treated with KCl-Ringer (Ets and Boyd, 1934), and on nerves

subjected to various forms of local injury. The blocking temperatures have been from +8.0 to −4.0°C. The findings of Bahrmann, therefore, do not apply to this form of cold block, and even for the freezing block they are quantitatively valid only under conditions which insure supercooling.

Effects of adherent salt solution. As a routine procedure the preparations were left for a time in Ringer or other saline solutions after dissection, and some of them were soaked for as long as two hours before use. The nerves were blotted with filter paper before mounting, but enough fluid remained to have some effect on conductivity. We carried out four experiments in which pipiens nerves were rinsed for 30 to 40 seconds in 4.5 per cent glucose solution before they were placed on the electrodes. The initial resistance of these nerves was comparatively high, but the form of the freezing-block curve was similar to that of the usual Ringer-soaked preparation. In three instances, after initial measurements of resistance on Ringer-soaked nerves at room temperature, isotonic glucose solution was applied with a pipette. This procedure, when brief and limited to the inter-electrode region, changed the resistance very slightly. Application at the points of contact with the electrodes caused a sharp rise (cf. Labes and Lullies, 1932).

Effect of removing the nerve sheath. On two bullfrog nerves the epineurium was slit for a length of about 4 cm., and reflected so that the bare nerve made contact with the electrodes. This was done with as little direct damage to the nerve as possible, but the affected segment in both cases was abnormally sensitive to cold. One was blocked at 3.0°, the other at 8.0°, with no break in the resistance curve. The nerves were removed from the moist chamber and left for 30 minutes in Ringer's solution. On cooling the same segments a second time, the blocking temperatures were respectively −6.1° (nerve frozen) and −4.0°. The mated preparations were mounted directly after dissection, without injury to the sheath, and both conducted until frozen. It was noted by Boyd and Ets (loc. cit.) that the cold blocking temperature tends progressively to rise near the point of section in a nerve, and that the effect is reversible in Ringer's solution. It is evident that relatively mild injury to the nerve impairs conduction at low temperatures.

Heat block. On four bullfrog nerves we obtained a perfectly reversible block at 39° to 41°. It was not associated with any break in the temperature-resistance curve, which is in agreement with the finding of Bremer and Titeca (1934) on another species. We also observed in each case the phenomenon of "Gewöhnung" described by Thörner (1920), a second heat block requiring a temperature 0.5° to 1.3° higher than the first. Heat of this degree produces an effect similar to that of mechanical injury, so that a subsequent cold block can be produced at a comparatively high temperature.

DISCUSSION. It seems clear that the extreme rise of resistance reported by Bahrmann is not a necessary condition for the failure of impulse conduction in cooled nerve. The resistance change does, however, appear to be of some significance in relation to the reversibility of the block. After a freezing block at near $-6.0°$ the nerve recovers only on long soaking in Ringer's solution, or not at all (Boyd and Ets, loc. cit.). The freezing block without supercooling involves a relatively small resistance change, and it is rapidly reversible. Chambers and Hale (1932) noted that in skeletal muscle the sarcolemma acts as a barrier to the spread of ice crystals. With the interstitial fluids frozen, the muscle fibers could still be subcooled through several degrees, with return of irritability on warming. It seems probable that a similar condition in nerve may be responsible for the reversibility of the freezing block at higher temperatures.

SUMMARY

1. The three- to five-fold increase of electrical resistance, reported by Bahrmann to be associated with cold block in frog nerve, is found to apply only to nerves frozen after supercooling to an average temperature of $-6.0°$C. A freezing block can be induced in normal frog nerve at -1.0 to $-2.0°$, with a resistance increase of less than 100 per cent.

2. The non-freezing cold block is not associated with any gross break in the temperature-resistance curve. This is also true of heat block, as previously reported by Bremer and Titeca.

3. Injury, such as is involved in careful dissection of the sheath, renders the nerve locally more susceptible to cold. This effect is reversible in Ringer's solution.

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ELECTRICAL ACTIVITY OF HUMAN MOTOR UNITS DURING VOLUNTARY CONTRACTION

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Sherrington (17) defined a motor unit as "an individual motor nerve-fibre together with the bunch of muscle-fibres it activates." Eccles and Sherrington (12) and Clark (6) (7) presented evidence which indicates that a single motor neurone may control 100 or more muscle fibers. Adrian and Bronk (3) (4) and Denny-Brown (11) first succeeded in isolating the motor unit as a functional entity.

Adrian and Bronk (4) studied reflex contractions in cats and voluntary contractions (mainly triceps) in man and concluded that the latter like the former are maintained by impulses which range in frequency from 5 to 50 or more per second in each nerve fiber. They further pointed out that gradation of strength of contraction is brought about by changes in the frequency of discharge of each unit and by the number of units active.

Smith (18) studied the motor unit responses in the biceps and triceps muscles of 8 normal subjects during voluntary contraction. Her results agreed essentially with those of Adrian and Bronk. She found no frequency of response above 20 per second but was unable to deal with strong contractions. During sustained contractions she found that a unit might remain continuously active for approximately 15 minutes without evidence of rotational activity.

In the present study an attempt has been made to determine the range of frequency of response of which an individual unit is capable during voluntary contraction, the manner in which gradation of strength of contraction occurs in various muscles and the effect of sustained contractions and fatigue. Records have been obtained from the muscles of 6 normal adult subjects, but for the most part from several representative flexor and extensor muscles of the writer. The muscles studied were the deltoid, biceps, flexor digitorum sublimis, brachio-radialis, rectus femoris, vastus lateralis, sartorius, gastrocnemius and tibialis anticus.

APPARATUS AND METHODS. For most of the work a six stage, transformer-coupled amplifier was used to drive either a loud speaker or a Du Bois oscillograph. A con-

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denser-coupled amplifier and a cathode ray oscillograph (see Gasteau and Davis, 14) have also been employed from time to time to check various points.

Four types of electrodes have been used. 1. A fine insulated wire (gauge no. 36) cemented into the lumen of a hypodermic needle (no. 20 or 21) with black baking varnish. The bared tip of the insulated wire led to grid and the outer portion of the needle to ground. 2. Two fine insulated wires inside a hypodermic needle, the bared tips of which were only a fraction of a millimeter apart, led to the primary of an input transformer. The needle was grounded. 3. A fine insulated wire (gauge no. 36) with a minute portion of its insulation scraped off was drawn through a muscle by means of a surgical needle until the bared portion made contact with an active unit. This wire led to grid, and a small metal plate covered with canton flannel soaked in NaCl solution was attached to the skin surface over an inactive region and led to ground. 4. Two fine wires with a knife-edge cut in the insulation were drawn through a muscle together and led to an input transformer.

Electrodes of type 1 served adequately for weak or moderate strengths of contraction but during strong contractions picked up responses from many neighboring units. Electrodes of type 2 were highly selective and served well for strong contractions but required greater amplification and were sensitive to movement. Types 3 and 4, although not easily applied in many muscles, were highly selective and less sensitive to movement than the needle electrodes. When the wires were in a muscle it was possible to move freely without discomfort or fear of displacement.

The needle electrodes were sterilized by autoclaving or soaking in 95 per cent alcohol. They were inserted to various depths in the muscle substance and held in place by a holder capable of tridimensional adjustment. The needles were inserted without anesthesia and except for the initial piercing of the skin did not cause discomfort.

A torsion wire myograph (mechanograph) was used for measuring the strength of contraction in the flexor digitorum sublimis muscle. The shadow of the myograph lever, which was attached to the middle finger, fell upon the film so that an upward deflection of the line indicates increasing tension. For roughly measuring the strength of contraction in the other muscles, hinged levers were arranged to pull against weights hung over a pulley.

RESULTS. When needle electrodes (type 1) were inserted in a muscle which was slightly tense, a rhythmic response was heard in the loud speaker as the electrodes reached the vicinity of an active unit. If the electrodes were inserted deeper or moved about in the muscle the response became louder or fainter depending upon the distance of the recording surfaces from the active unit. In a muscle which was quite tense other units were heard as sounds of slightly different character, indicating that more than one unit was active in the immediate neighborhood of the electrodes.

Most normal subjects can relax a muscle so completely that, although full amplification is used and the electrodes are inserted in different parts of the muscle, no active units are found. Relaxation sometimes requires conscious effort and in some cases special training, although in none of the 6 normal subjects used in this study was the complete relaxation of a muscle difficult.

The recorded responses of a single motor unit (fig. 1) during a voluntary

contraction are characterized by a distinctive pattern,[2] which serves to distinguish them from the responses of other units, uniform amplitude and a fairly regular rhythm. The rhythm in this case varies by approximately 10 per cent of the average interval between successive responses, which is the usual amount of variation. In some cases, however, the rhythm may vary by as much as 30 per cent (see fig. 4 A). In general the rhythm is less regular during weak than during moderate or strong contractions.

The frequency range of motor unit responses during voluntary contraction did not vary significantly in different parts of the same muscle or in the several muscles studied, with the exception of the biceps in which the upper frequency limit was on the average a little higher than in the other muscles. Motor units usually began to respond regularly at frequencies of 5 to 10 per second during the weakest voluntary contractions which could be made, although occasionally a unit began to discharge at a rate of 12 to 15 per second if a number of other units were already active. It was sometimes possible to obtain regular responses at a frequency as low as 3 per second if an attempt was made to slow down a unit which was already discharging. Usually, however, when such attempts were made the responses became irregular and ceased entirely.

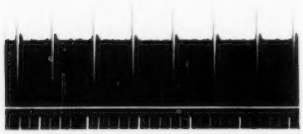

Fig. 1. Action potentials from a single motor unit in the rectus femoris muscle of a normal subject during a weak voluntary effort (extension). Frequency, 13 per second. Time units equal 0.020 second.

When the strength of contraction was gradually increased, the frequency of response of the motor units increased and at the same time more and more units throughout the muscle became active. This is illustrated in the records in figure 2, which were taken during a series of contractions of increasing strength in the biceps muscle. Record A was obtained during the weakest voluntary contraction which could be initiated and shows a unit responding fairly regularly at a frequency of 5 to 6 per second. Record B shows the frequency increased to 9 per second and C to 11 per second with the accession of a new unit. With still stronger contractions the frequency of each unit increased further and responses of other units appeared.

It is difficult to evaluate the part played by the two mechanisms (change of frequency in each unit and variation in the number of units active) for grading the strength of a contraction, since both operate throughout the

[2] A transformer-coupled amplifier of the type used for obtaining most of the records distorts the wave-form of the action potential, but does not interfere with the identification of individual units by their general pattern which is determined largely by the relation of the recording surfaces of the electrodes to the active units.

entire range. In one experiment during a very weak contraction of the flexor digitorum sublimis (a small muscle) slow rhythmic responses (5 per second) of a motor unit were correlated with slight undulations of the myograph curve of tension recorded from the middle finger. This indicates that there was probably only one unit active in the entire muscle and that there was not complete summation of contractile effect. In weak contractions, when the frequency of response in each unit is low, smoothness of the contraction depends upon the asynchronous response of several units. Since the frequency of response in individual units increases quite

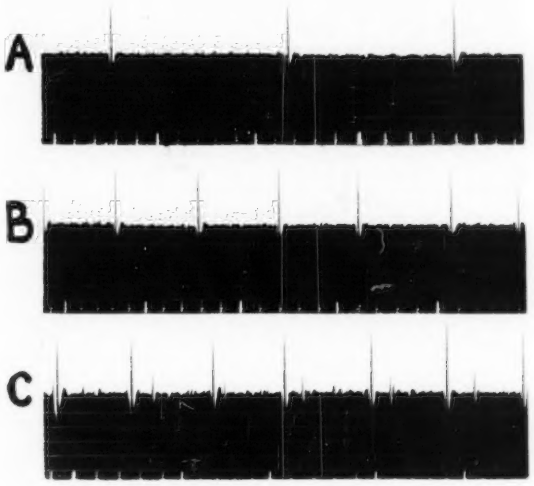

Fig. 2. Motor unit responses from biceps muscle of normal subject during increasing voluntary effort (flexion). A, weakest possible effort, frequency, 5 to 6 per second; B, stronger effort, frequency, 9 per second; C, still stronger effort, frequency, 11 per second and accession of a new unit at 10 per second. Time units equal 0.023 second.

evenly with gradually increased effort, whereas the accession of each new unit represents a discrete step, it appears that the change in frequency is the more delicate method of grading the strength of contraction. The accession of units is probably a quicker and more potent factor in increasing the strength of a contraction.

During the ordinary range of voluntary muscular activity, including weak to moderately strong contractions, the frequency of motor unit responses does not exceed 30 per second. Only during very strong contractions involving maximal or near maximal effort does the frequency of

response become higher. As Adrian and Bronk (4) have pointed out, it is difficult to study the upper frequency limit of response of single motor units during very strong contractions because of the swamping of the rhythm by other units. With electrodes of types 2 and 4 we have found it possible to isolate and record the response of single motor units in several muscles during maximum effort. The highest frequencies of response we have found in the muscles of normal subjects were in the neighborhood of 40 per second. Figure 3 C shows responses of a single motor unit in the biceps muscle during the strongest voluntary contraction the subject could make. The frequency of the responses is on the average about 40 per second, although the highest frequency attained by any two successive responses is approximately 45 per second.

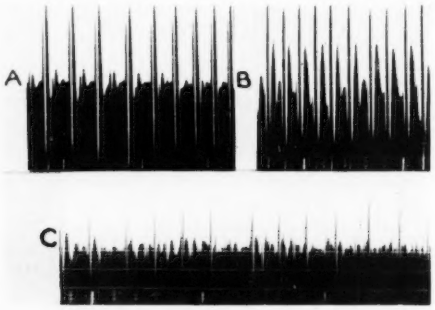

Fig. 3. Action potentials from single motor units during the strongest voluntary effort possible. A and B from biceps muscles of patient with progressive muscular atrophy, frequencies, 30 to 40 and 45 to 50 per second, respectively. C from biceps of normal subject, frequency, 40 per second. Time units equal 0.023 second.

Patients with progressive muscular atrophy have provided a rather unique situation for investigating further the upper frequency limit of the motor unit response during voluntary contraction. In several such patients the biceps and triceps muscles were atrophied to such an extent that only a small strand of muscle tissue remained. On examination it was found that the few remaining units in these muscles appeared to function normally. It was possible therefore to study maximal voluntary effort in these units without interference from other units. Figure 3 A and B are records from different units in the severely atrophied muscles of one of these patients during an attempt to flex the arm at the elbow. In A a motor unit responds at a frequency of 30 to 40 per second and in B at 45 to 50 per second. The biceps of this patient was only about as large in diameter as his little finger and he was not able to flex the arm against gravity.

On several occasions a single motor unit has been kept continuously active for periods ranging from 15 to 30 minutes during a sustained contraction which did not cause fatigue. Records were taken at regular intervals during the course of the contraction and the unit's response followed by ear with the loud speaker between records. Figure 4 A was taken at the start of a sustained contraction of the tibialis anticus muscle and B 30 minutes later. Throughout the duration of the contraction the unit was continuously active and there was no evidence of the substitution of one unit for another. The records show that the frequency and amplitude of the motor unit responses remained the same.

Records of single motor unit responses have also been obtained during strong sustained contractions which produced fatigue within a few minutes. These records show progressive decrease in amplitude of the motor unit responses as fatigue develops. Figure 5 shows a series of records from the tibialis anticus muscle of a normal subject during a strong contraction maintained as long as possible. Record A was taken at the start of the contraction. The single motor unit responses are of uniform amplitude and at a frequency of 9 per second. Record B, obtained 4 minutes later when fatigue was first felt, shows the unit at the same frequency but with a slight diminution of the amplitude of the responses. Record C, obtained 1 minute later and just prior to complete fatigue, shows the unit responding at approximately the same frequency but with greatly diminished amplitude.

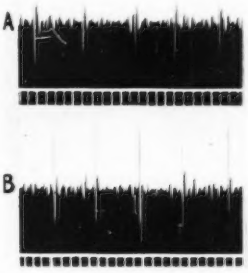

Fig. 4. Action potentials from a motor unit in tibialis anticus muscle during a sustained contraction (weak), showing no variation in amplitude or frequency of response. A, at start; B, 30 minutes later. Time units equal 0.020 second.

Further confirmation of the decrease in amplitude of the motor unit responses with fatigue has been demonstrated in the muscles of the patients with progressive muscular atrophy. Figure 6 shows records obtained from the flexor digitorum sublimis muscle of the forearm, which was greatly atrophied. The middle finger was attached to a myograph lever and a constant voluntary contraction maintained until fatigue ensued. In record A, taken at the start, a motor unit responds at a frequency of 13 per second. Record B taken 5 minutes later, when fatigue was very marked, shows reduced amplitude of the motor unit responses, although the frequency remains approximately the same. One also notes irregularities of the myograph curve resulting from fatigue.

Some information about the relative size of the groups of muscle fibers

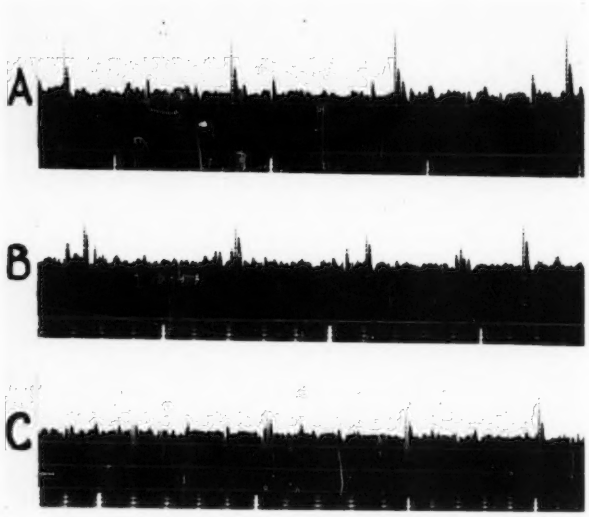

Fig. 5. Action potentials from tibialis anticus muscle during a strong contraction, showing progressive diminution of amplitude with fatigue. A, at start; B, 4 minutes later, C, 1 minute later and just prior to complete fatigue. Time units equal 0.023 second.

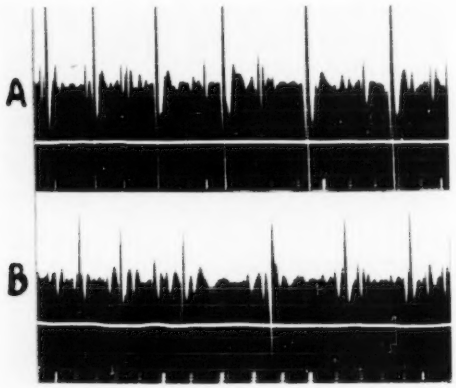

Fig. 6. Motor unit responses from flexor digitorum sublimis muscle of a patient with progressive muscular atrophy during a contraction which caused fatigue. A, at start; B, 5 minutes later, showing reduced amplitude of response. White line indicates tension against myograph. Time units equal 0.023 second.

which compose a unit and the distribution of the fibers has been obtained with the highly localizing electrodes (types 2 and 4). The movement of the electrodes a millimeter one way or the other often completely eliminated the response of a unit, thus indicating that the diameter of such a group of fibers is somewhere in the neighborhood of one millimeter. Adrian (1) has shown that a nerve fiber may divide and send branches to both halves of the tenuissimus muscle of the cat, but for the most part the muscle fibers which compose a unit are probably not widely separated. Our experience indicates that muscle fibers of a unit are grouped, possibly in longitudinal chains, as Cooper (8) showed, rather than widely distributed throughout a muscle. If such is the case, it would make the response of a single active unit effective, as we have observed, and would result in greater effectiveness of all units during sub-maximal contractions.

DISCUSSION. Of particular interest is the fact that the frequency of motor unit responses during voluntary contraction did not exceed 40 to 50 per second and for the most part fell within the range of 5 to 30 per second. Although it may be possible that the frequency of response could be increased still further during so-called "super-human" feats of strength, the strongest voluntary effort that could be produced in an experimental situation would not drive motor units at frequencies above 50 per second.

In view of the findings of Adrian and Bronk (3) (4) that the frequency of impulses in individual fibers of the phrenic nerve of the cat ranged from 15 to 90 per second, it might have been expected that motor units generally would be capable of such a range. However, in single nerve fibers to other muscles of the cat they found the highest frequencies per second during reflex excitation were as follows: peroneus longus, 30; tibialis anticus, 44; quadriceps, 90; vastus lateralis, 25; vastus medialis, 65. They also demonstrated that stimulation of the whole nerve to the gastrocnemius and tibialis anticus produced curves of tension which showed but little increase in response to stimulating frequencies above 50 to 60 per second. Liddell and Sherrington (15) and Cooper and Eccles (9), on the other hand, have shown that stimuli at the rate of 90 per second or more are required in some cases to produce a completely smooth contraction. It would appear therefore that some muscles require impulses in the neighborhood of 70 to 90 per second to produce a completely fused tetanus, whereas other muscles require frequencies of only 30 to 50 or 60 per second. However, during normal reflex or voluntary excitation muscles probably seldom produce a completely fused contraction.

It is interesting that action potentials of single motor units recorded from muscles during reflex contractions (4) (11) (10) have not apparently exceeded 20 to 30 per second in most cases. Similarly the responses of single motor units recorded from the intercostal muscles of the cat during normal inspiration by Adrian (2) and during hyperpnea by Anderson

and Lindsley (5) have not exceeded rates of 15 to 30 per second. These examples as well as the highest frequencies of response found during voluntary contraction of human muscles in this study fail to correspond with the highest frequencies of response reported in the individual nerve fibers of the phrenic and elsewhere by Adrian and Bronk (3) (4).

A point which calls for further explanation is the fact that a motor unit during fatigue (fig. 5) shows a progressive diminution of amplitude whereas a motor unit during sustained contraction (fig. 4), which does not cause fatigue, shows no variation in amplitude or frequency of response, although it discharges at the same rate and for a longer period of time. The difference might be accounted for in terms of the available oxygen supply, which is proportionally less per unit in the former case, in which the contraction is much stronger and a greater number of units are active. Therefore there is probably a greater accumulation of lactic acid and metabolites in the fatigued muscle. It is also probable that the blood flow is less during a strong contraction than a weak one.

Fatigue in normal subjects does not appear to be of the same type as the weakness and fatigability of the muscles of patients with myasthenia gravis, for in the latter Lindsley (16) demonstrated not a uniform and progressive decrease in amplitude of motor unit responses but irregular amplitude from the start.

Both Denny-Brown (11) and Adrian and Bronk (4) have failed to find evidence of rotational activity of motor units during sustained reflex contractions. The present evidence, as well as that presented by Smith (18), on voluntary contractions in man has likewise failed to substantiate Forbes' (13) original suggestion of the rotational activity of units as an explanation of the lack of fatigue in long-sustained contractions.

SUMMARY

Action potentials have been recorded from single motor units in several flexor and extensor muscles of 6 normal human subjects during various strengths of contraction, sustained effort and fatigue.

No electrical activity has been demonstrated in any part of a relaxed muscle. During a voluntary contraction the response of a motor unit is characterized by a distinctive pattern of uniform amplitude and fairly regular rhythm. The rhythm may vary by as much as 10 to 30 per cent during a constantly maintained voluntary effort.

The two means by which the strength of contraction may be graded, namely, change in frequency of discharge in each unit and in number of units active, operate together throughout the range of contraction intensities. Change in frequency is probably the most delicate grading mechanism and change in number of units the most effective.

The lowest regular frequency of response found during voluntary con-

traction was 3 per second, the highest frequency 50 per second. During the weakest voluntary contraction which could be initiated, motor units usually began to respond at frequencies between 5 to 10 per second. With the exception of the upper frequency limit, which was slightly higher in the biceps than in the other muscles, motor unit responses did not differ significantly from one muscle to another or in different parts of the same muscle.

Motor units kept continuously active for 15 to 30 minutes during a weak sustained contraction showed no sign of rotational activity or variation in frequency and amplitude.

During fatigue produced by strong, constantly maintained contractions, single motor unit responses progressively diminish in amplitude but maintain the same frequency.

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THE CHEMICAL TRANSMISSION OF VAGAL EFFECTS TO THE SMALL INTESTINE[1]

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Since the initial work of Loewi demonstrating the chemical transmission of the cardiac inhibitory effect of the vagus similar transmissions have been described for other parasympathetic mechanisms as well as for conduction across sympathetic ganglia. A chemical substance, most likely if not undoubtedly acetylcholine, has now been reported after stimulation of parasympathetic nerves to the eye (4), the salivary glands (1), the tongue (5), the bladder (7), and the stomach (2). To this lengthening list we wish to add the vagus nerves to the small intestine.

METHODS. A. *Denervated intestinal loop as indicator.* Our interest in the problem of chemical transmission of parasympathetic impulses arose from some experiments which were being carried out on the effect of various substances on denervated intestinal loops in recovery animals. It occurred to us that such denervated loops might serve as an indicator for the presence of a humoral agent following vagal stimulation. Since both electrical stimulation of the nerves and injection of eserine were necessary the experiments were made acute and carried out on dogs anesthetized either with chloralose or more usually with about 200 mgm. per kilo of sodium barbital administered intravenously. Two loops, each about 10 cm. long, were made just below the ligament of Treitz. One of these was completely denervated by removal of all the mesenteric tissue except the vein and artery which were burned with phenol and then washed with alcohol and salt solution. The activity of each loop was recorded by the usual balloon tambour method. Artificial respiration was then installed and the vagus nerves exposed just above the diaphragm. The nerves were severed and the distal ends placed in shielded electrodes which were connected to a Harvard induction coil. At the appropriate times eserine or atropine were injected intravenously.

B. *Intestinal perfusion.* Although positive results were secured by the method just described it was obvious that the substance stimulating the denervated loop might have originated in any region innervated by the

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vagus. It was at this stage of our work that Dale and Feldberg (2) reported acetylcholine from the stomach. We therefore decided to perfuse the small intestine.

Dogs anesthetized with sodium barbital received an intravenous injection of 1 mgm. per kilo of eserine hydrochloride which was designed to inhibit the tissue cholinase. It is probable that this may have been unnecessary since Feldberg and Kwistkowski (6) found that eserine in the perfusion fluid was sufficient for this purpose. The animals were then bled to death and during this procedure ligatures were tied around the coeliac and superior mesenteric arteries. The perfusion cannula was inserted in the mesenteric artery above the ligature. Any perfusion by way of anastamoses was prevented by ligatures around the upper end of the colon and the lower part of the duodenum. The outflow cannula was placed in the portal distal to the entrance of the gastric, splenic and pancreatic branches.

The perfusion fluid was a modified Locke's solution consisting of NaCl 0.9, $CaCl_2$ 0.016, KCl 0.0207, $NaHCO_3$ 0.022 and glucose 0.1 all in per cents. On the addition of 0.38 cc. of distilled water to each cubic centimeter of perfusate it was diluted properly for the cold blooded heart. The perfusion of the intestine was carried out by the gravity method, the fluid being kept at 36°C. and completely oxygenated. By means of large and small mariotte vessels the perfusion pressure was kept constant and a known amount of the solution to be tested could be delivered to the perfusion cannula.

Tests for the presence of an acetylcholine like substance were made on the frog's heart, leech muscle and the frog's rectus abdominis. The latter was not sensitive enough for our purposes. Perfusion of the frog's heart with Ringer's through its left auricle proved most satisfactory, the heart responding quite uniformly to acetylcholine controls as well as the perfusates.

RESULTS. A. *Denervated loop as indicator.* If stimulation of the vagi above the diaphragm releases an acetylcholine like substance in the splanchnic area, and this is protected by eserine, it should enter the circulation and shortly reach the denervated loop causing its musculature to respond beyond the usual small rhythmical contractions. That this occurs may be seen from figure 1. The first stimulation of the vagus caused only the innervated loop to respond. The denervated one of course could not be reached nervously and any A-C like substance was destroyed by blood and tissue esterase before reaching the loop. The second stimulation occurred after a small injection of eserine. The innervated loop again responded and some 30 seconds afterward the denervated loop also contracted. Obviously the mechanism involved must have been a humoral one. Since it followed parasympathetic stimulation, appeared only after eserine and was abolished by atropine, it is reasonable to conclude that we were dealing with acetylcholine or at least a similar substance.

In every experiment such results were occasionally secured and in several they were repeated frequently. The difficulty in all cases seemed to be in the injection of just the right amount of eserine. Too much threw both loops into series of tonic contractions and one could not be sure of the relationship between the two curves. After small doses however the musculature often remained at its usual level of activity and then results as described were observed frequently enough to be convincing.

B. *Intestinal perfusion.* Since our results so far only showed the production of an A-C like substance somewhere in the splanchnic area we

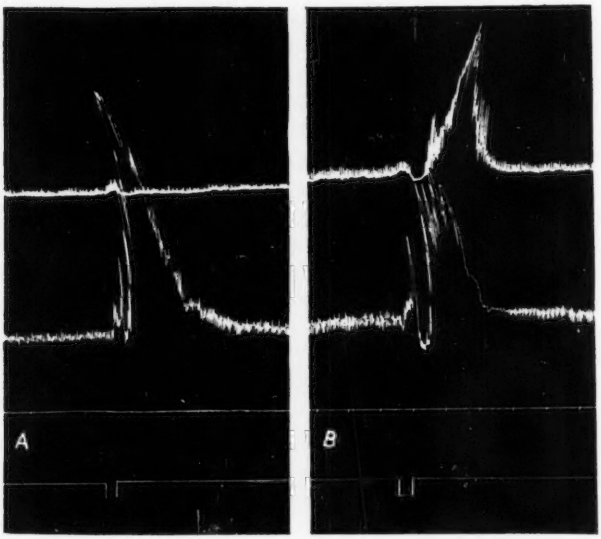

Fig. 1. Record from denervated loop above and normal one below. Time in seconds. Lower line marks vagal stimulation. Between A and B a small dose of eserine, 0.3 mgm. was injected intravenously.

decided to perfuse the intestine and find if it was one of the producing organs.

Perfusions as described were therefore made. The gut was first perfused at a rapid rate for about 15 minutes which was long enough to wash out all the blood and to allow the spontaneous production of the A-C like substance to come to a constant level. The perfusion rate was then reduced to about 1 cc. per minute and the control samples collected. Tetanic vagal stimulation from a Harvard coil in alternating 5 second periods was then instituted and the samples collected during the next 20 or 30 minutes. All samples received 0.2 cc. of a 1/1000 solution of eserine for each 10 cc.

and were diluted with distilled water for the cold blooded heart. They were kept at a temperature of about 15°C. until tested. As shown by Feldberg and Kwistkowski (6) even during perfusion a depressor substance is continually being formed in the splanchnic area. Our records have always confirmed these findings. To judge whether vagal stimulation has been effective the perfusate after stimulation must be compared with the control. In figure 2 may be seen the results of one striking experiment. Although the control contained enough A-C like substance to decrease the contrac-

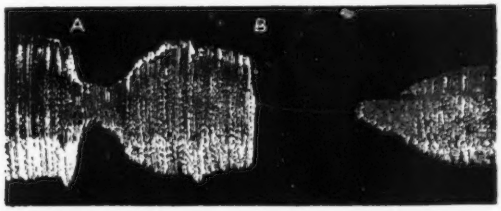

Fig. 2. Record from perfusion of frog's heart. At A 1.4 cc. of the control perfusate. At B, 1 cc. of perfusate after vagal stimulation.

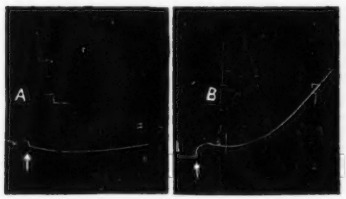

Fig. 3

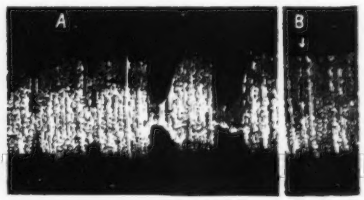

Fig. 4

Fig. 3. A is the response of eserinized leech muscle to control and B to the perfusate secured during vagal stimulation. The immersion in the solutions lasted exactly 3 minutes.

Fig. 4. Frog's heart. A shows two responses each to control intestinal perfusate and perfusate after vagal stimulation. At B is a response of the atropinized heart to the same intestinal perfusate taken during stimulation.

tions of the frog's heart markedly, a still smaller amount of the perfusate secured during stimulation completely abolished the beat.

The exact amount of A-C like substance in the perfusates is estimated only with considerable difficulty, in our opinion the frog's heart not being very suitable for such determinations. In one experiment, however, with rather satisfactory results it was found by using known solutions of acetylcholine that the control perfusate had a strength of about $1:10^8$ A-C like substance and the stimulated about $1:10^7$ or ten times as much.

In figure 3 evidence for the function of an A-C like substance during vagal stimulation is shown by the use of the leech muscle as a test object. The procedure used was that described by Dale and Feldberg (3) and others.

It is well known that atropine abolishes the response of the frog's heart to acetylcholine. Figure 4A very strikingly shows the increase in response to intestinal perfusates taken during vagal stimulation. This response was almost but not quite entirely abolished when the heart was atropinized as may be seen in figure 4B. This is evidence that we are dealing either with acetylcholine or an A-C like substance. The fact that the depressor effect of the intestinal perfusate did not entirely disappear after atropine probably speaks for a certain resistance to atropine or a small amount of some other unknown substance, which seems to increase along with the vagal stimulation. Histamine might be thought of in this connection. The inhibition from the control was always greater than the residual depression remaining after the test heart was atropinized. The control intestinal perfusate therefore also always contained an A-C like substance.

COMMENT. For the following reasons our results lead us to believe that the substance appearing in the intestine during vagal stimulation is acetylcholine: 1, its stimulation of contraction of a denervated intestinal loop; 2, its inhibitory action on the frog's heart is similar to that of acetylcholine; 3, its stimulating action on the eserinized leech muscle is comparable to that of acetylcholine; 4, the abolition of its action by atropine; 5, its protection by the addition of eserine to the perfusate; 6, its disappearance on standing at room temperature.

The demonstration of acetylcholine on vagal stimulation of the intestine adds another example of chemical transmission of peripheral parasympathetic effects.

The demonstration of acetylcholine in an organ such as the intestine in which atropine does not entirely inhibit the augmentor action of the vagus, strengthens the current conception that the transmitter of parasympathetic impulses is acetylcholine. So far there is no evidence as to the exact site of its origin in the intestine.

SUMMARY

By the use of an intestinal loop in an intact animal as an indicator the production of an acetylcholine like substance was shown to arise in the splanchnic area during vagal stimulation. That this substance came in part at least from the small intestine was demonstrated by perfusion of this organ and tests on the perfusate. To the perfusate both the frog's heart and the eserinized leech responded in a manner typical for acetylcholine. The material could be demonstrated only when protected by eserine, its action was almost entirely abolished by atropine and it disappeared on standing. It is therefore believed to be acetylcholine like and most likely

acetylcholine itself. The demonstration of this chemical transmitter of parasympathetic impulses in an organ like the intestine which is somewhat atropine resistant is of considerable significance.

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"HUNGER DIABETES" AND THE UTILIZATION OF GLUCOSE IN THE FASTING DOG

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The previous literature on the hyperglycemia and glycosuria which results from carbohydrate ingestion following a fast or a diet deficient in carbohydrates has been reviewed by Dann and Chambers (1930). These workers confirmed the occurrence of these "diabetic" manifestations in the fasting normal dog and, in addition, showed that the initial administration of glucose under these circumstances caused little or no rise of the non-protein respiratory quotient from the low resting level at which it is maintained during fasting. They concluded that there is almost complete suppression of the ability to oxidize ingested glucose in dogs after a fast of about 3 weeks.

We have recently shown (Soskin, et. al., 1934a, 1934b, 1935) that tolerance to administered sugar cannot be related to the insulinogenic activity of the pancreas or to glucose utilization by the muscles, but is, rather, a function of the liver. On the basis of our results the manifestations of "hunger diabetes" are best explained as a delay in the normal homeostatic reaction of the liver, whereby this organ decreases its supply of glucose to the blood in response to an influx of exogenous sugar. From this point of view, neither the low R.Q. of fasting nor the failure of sugar administration to raise this R.Q. should be interpreted as indicating a suppression of glucose oxidation. It therefore seemed worth while to investigate the utilization of carbohydrate by the fasting dog, using a criterion other than the respiratory quotient.

METHODS. Our procedure consisted in observing the effect of previous fasting on the rate of disappearance of the blood sugar in dogs after complete evisceration. The operations were performed under nembutal anesthesia by a modification of the method for hepatectomy described by Markowitz, Yater and Burrows (1933). For two hours following the operation, arterial blood samples were drawn at frequent intervals and analyzed for sugar content by the Somogyi modification of the Shaffer-Hartman method. Dextrose was then administered intravenously and the

[1] Aided by the Max Pam Fund for Metabolic Research.

observations repeated. The ureters were ligated in all cases to obviate loss of sugar by excretion. Some blood lactic acid determinations were also made at various stages of the experiments.

The periods of fasting which we utilized ranged from 18 to 38 days, in accordance with the conclusions of Dann and Chambers (1930) as to the period required for complete suppression of glucose oxidation. Our control animals were fasted for 48 hours in order to facilitate the operative procedure and post-operative survival. This short period of fasting, according to previous workers (quoted by Dann and Chambers, 1930), is not followed by the manifestations of "hunger diabetes."

TABLE 1

DOG NUMBER	PERIOD OF FASTING	DISAPPEARANCE OF BLOOD SUGAR FOLLOWING EVISCERATION					DISAPPEARANCE OF BLOOD SUGAR IN SAME EVISCERATED ANIMALS FOLLOWING DEXTROSE ADMINISTRATION				
		Immediate	15 min.	30 min.	60 min.	120 min.	15 min.	30 min.	60 min.	90 min.	120 min.
	days	*mgm. per cent*	*mgm. per cent*	*mgm. per cent*	*mgm. per cent*	*mgm. per cent*	*mgm. per cent*	*mgm. per cent*	*mgm. per cent*	*mgm. per cent*	*mgm. per cent*
Control 1	2	81	70	65	57	32	219	178	136	124	120
Control 2	2	78	54	48	31	20	217	175	134	115	99
Control 3	2	93	85	67	47	35	252	199	143	—	—
Control 4	2	76	55	47	48	27	105	89	64	44	48
Control 5	2	66	53	45	23	16	155	122	106	86	60
Control 6	2	72	60	52	41	28	95	74	46	38	18
Fasting 1	18	68	49	32	13	19	177	144	104	84	63
Fasting 2	21	61	53	46	36	11	109	97	78	61	38
Fasting 3	21	75	62	55	41	33	131	96	82	51	42
Fasting 4	22	73	55	50	43	27	184	139	105	89	72
Fasting 5	25	78	53	46	32	17	185	153	141	—	—
Fasting 6	38	45	25	15	10	8	154	129	120	88	69

RESULTS. The results are detailed in table 1 and graphically summarized in figure 1. It may be seen that the rate of disappearance of the blood sugar after evisceration in the fasted animals is just as rapid as in the controls. This applies to administered dextrose as well as to the blood sugar of endogenous origin.

In neither the fasted nor the control animals, either before or after the administration of dextrose, does the accumulation of lactic acid in the blood account for a significant proportion of the blood sugar which disappears.

It is of interest to note that the absolute rate of fall of the blood sugar in the individual experiments (table 1), both in the fasted and control dogs,

depends on the initial blood sugar level. When, however, this difference in initial height is obviated by averaging the values for all the experiments of each group and charting these values as percentages of the initial level (fig. 1), the rate of disappearance of blood sugar in all the experiments coincides within narrow limits.

Discussion. Once the liver is removed it becomes quite evident that the extrahepatic tissues of the fasting dog dispose of sugar just as rapidly as do those of the control dog. Unless one is to make the entirely unwarranted assumption that evisceration causes an immediate and radical change in the material oxidized by the remaining tissues, one is forced to

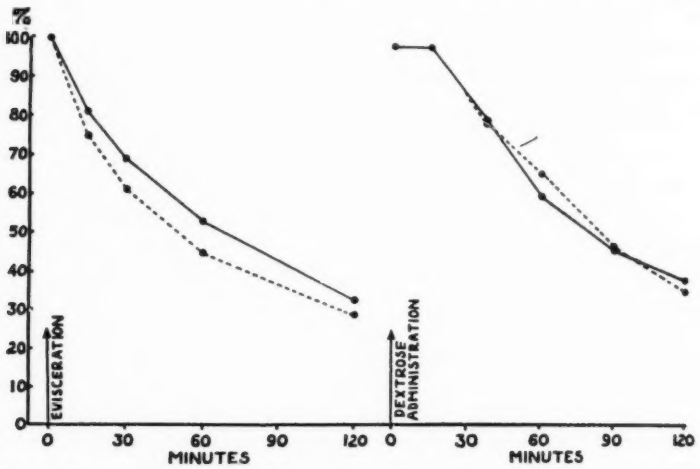

Fig. 1. Graphic comparison of the rates of disappearance of blood sugar after complete evisceration in the fasting (broken lines) and control (solid lines) dogs, with and without sugar administration. Each curve represents the average values for all the experiments of the particular group, plotted as the percentage of the initial average blood sugar level.

the conclusion that the intact fasting animal suffers no suppression of its ability to utilize carbohydrate. These results and conclusions are in complete accord with our previous considerations of "hunger diabetes." They further indicate that the low respiratory quotient of fasting cannot be interpreted as meaning a lack of carbohydrate oxidation.

The present work is but the latest of a series of observations, made within recent years, which indicate the fallacy of the generally accepted interpretation of the respiratory quotient. The utilization of sugar has now been demonstrated in practically every physiological preparation in which the value of the R.Q. is ordinarily interpreted to mean an inability to oxidize carbohydrate. Such evidence is available:

1. In the phlorhizinized dog (Wierzuchowski, 1926, 1927a, 1927b; Deuel, Wilson and Milhorat, 1927).

2. In the depancreatized dog, after depletion of its stores of body fat (Soskin, 1930; Ring, 1934).

3. After hepatectomy of the depancreatized dog (Mann and Magath, 1923).

4. During prolonged injections of epinephrin in the normal dog (Soskin, Priest and Schutz, 1934c.

5. After hypophysectomy of the depancreatized dog (Houssay and Biasotti, 1931).

6. After evisceration of the fasting normal dog.

The outline and bibliography of an alternative interpretation of the respiratory quotient has been discussed in a previous communication (Soskin, 1930). It is not our purpose to enlarge upon it at the present time. It seems important, however, to again point out the need for some revision of the present interpretation of the respiratory quotient.

CONCLUSIONS

1. As judged by the utilization of sugar after evisceration, there is no suppression of carbohydrate oxidation in the normal fasting as compared to the normal fed animal.

2. The low respiratory quotient of fasting as well as the other manifestations of "hunger diabetes" are not due to a lack of carbohydrate utilization, but probably result from an increased gluconeogenesis.

3. The need for a re-interpretation of the respiratory quotient is emphasized.

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INFLUENCE OF HYPOPHYSECTOMY ON GLUCONEOGENESIS IN THE NORMAL AND DEPANCREATIZED DOG[1]

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Among the numerous studies of Houssay and his collaborators (1) on the hypophysectomized organism are a number of observations which indicate a profound disturbance in the normal mechanism for the maintenance of the blood sugar level. Those observations which were made on the dog, and which have been partly confirmed on the same animal by subsequent workers, may be briefly summarized as follows:

1. The fasting, hypophysectomized dog shows a progressive drop in the blood sugar level, and may die in hypoglycemic convulsions (2) (3) (4) (5) (6).

2. The hypoglycemic effect of fasting persists after pancreatectomy of the hypophysectomized animal (1) and is exaggerated by phlorhizination (7).

3. The hypoglycemia which rapidly follows the administration of phlorhizin to the hypophysectomized dog is alleviated by the administration of carbohydrate or protein, but not by fat (7).

4. Pancreatectomy of the hypophysectomized dog is followed by a modified form of pancreatic diabetes. The animal may survive without insulin for months (1) (8) (9) (10). Similar results to the above have also been reported in a variety of animal species recently reviewed by Collip (11).

The above phenomena cannot but have an important bearing on the interpretation of the previously established facts concerning the origin of the blood sugar, and the metabolic disturbance in pancreatic diabetes and diabetes mellitus. With this in mind we have observed, since the early reports of Houssay et al., a series of hypophysectomized and of hypophysectomized-depancreatized dogs.

SOURCE MATERIALS FOR THE BLOOD SUGAR IN THE HYPOPHYSECTOMIZED DOG. It is well known that the starving normal dog maintains its blood

[1] Presented in preliminary form before the American Physiological Society, April, 1935.

[2] Aided by the Max Pam Fund for Metabolic Research.

sugar at an approximately normal level long after marked derangements in many other blood constituents have occurred and, indeed, up to the time of death. Although a small part of this phenomenon may be due to a diminished use of carbohydrate concomitant with the lower metabolism of the intermediate stages of inanition, it seems clear that the chief reason for the persistence of the normal blood sugar level is the continued production of blood sugar from the non-carbohydrate materials of the body. This view is supported by the fact that removal of the liver from a starving animal results in as rapid a fall of the blood sugar to hypoglycemic levels as occurs after the same procedure in the well fed animal (12).

The hypoglycemic effect of starvation in the hypophysectomized animal might be interpreted as being due to:

1. An increased consumption of sugar by the tissues, or
2. A decreased formation of blood sugar from endogenous non-carbohydrate sources.

The lowered metabolic rate following hypophysectomy and the fact that hypoglycemia also occurs in the fasting depancreatized-hypophysectomized animal are opposed to the first interpretation. The hypoglycemia is, therefore, more readily interpreted as being due to a failure of that mechanism which so well maintains the blood sugar supply of the fasting normal dog.

In the light of the above considerations, the fasting hypoglycemic hypophysectomized dog seemed to offer a suitable test-object upon which to determine the sugar-forming properties of the non-carbohydrate foodstuffs, by simple feeding experiments.

Methods. Adult dogs were hypophysectomized by a method slightly modified from that of Dandy (13). During the immediate post-operative period glucose was administered intravenously at frequent intervals until the animal could partake of food by mouth. When the dogs had recovered completely from the surgical interference and the wounds were healed, they were fasted for 24 hours in order to assure ourselves of the presence of the hypoglycemic effect in each animal. Only such dogs in which the blood sugar was 50 mgm. per cent or lower, after this short period of fasting, were used in this study.

Three sets of observations were made on each animal, viz., the blood sugar level when the animal's diet consisted solely of fat, when the diet consisted of protein, and when the animal was fasted for a number of days. The fat was administered in the form of olive oil, by stomach tube. Protein was administered as lean meat. The amount of each food given was more than that necessary for the animal's caloric requirement. The diets were divided into five equal portions which were administered at 9 a.m., 12 noon, 3 p.m., 6 p.m. and 9 p.m. Blood samples were taken before each feeding for the determination of the glucose content by the Somogyi modification of the Shaffer-Hartman method.

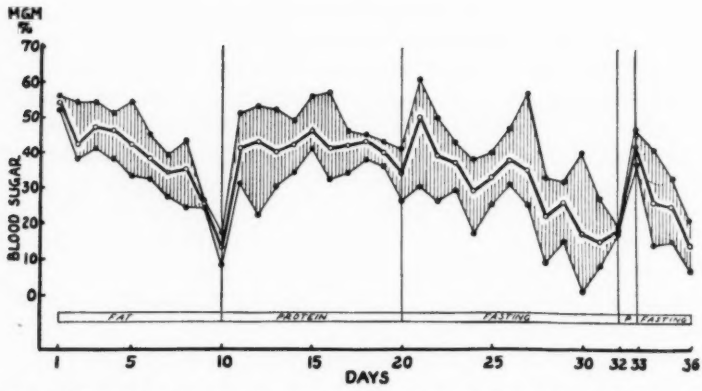

Fig. 1. Effect of exclusive fat or protein feeding and of fasting on the blood sugar level of the hypophysectomized dog. The shaded area represents the spread of the blood sugar values and was obtained by plotting the maximum and minimum blood sugar values for each day. The central heavy line indicates the average of all the blood sugar values obtained on each day.

In the experiment represented in this figure, the notations as to the material fed represent: Fat—11 grams per kilogram body weight per day, in the form of olive oil, by stomach tube; protein—11 grams per kilogram body weight per day, in the form of lean meat.

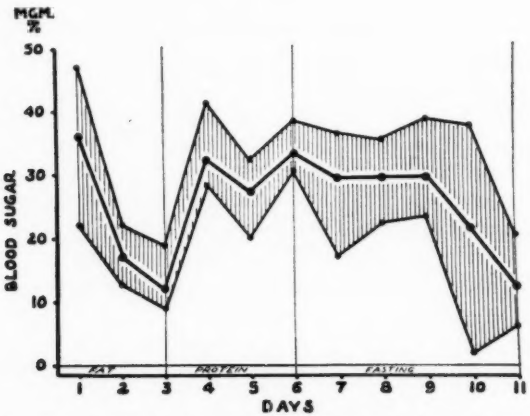

Fig. 2. Another experiment similar to that represented in figure 1, except that this animal went through the various stages much more rapidly than the other.

This animal received 15 grams fat per kilogram body weight per day, and 31 grams protein per kilogram body weight per day in the respective periods.

Results. The number of blood sugar determinations made in this study was such as to preclude detailed tabular presentation. Of the six animals studied in this way, two typical experiments are illustrated in figures 1 and 2. The blood sugar values for each day are charted as maximum, minimum and mean, in order to emphasize the trend of the blood sugar level from day to day rather than the fluctuations within each 24 hours.

It may be seen that in these experiments protein feeding maintained the blood sugar level and alleviated hypoglycemia when present, while the administration of even greater amounts of fat was without demonstrable effect as compared to starvation.

The experiments represented in figures 1 and 2 were selected to show the difference between different animals in the time required for the onset of hypoglycemia during either fat feeding or starvation. These periods not only differed in different animals but also varied in the same animal at different times, depending upon the previous feedings and the current state of nutrition. It may be seen in figure 1 that after a rather prolonged fasting period and a rapid recovery of the blood sugar level by the administration of protein for one day, a second fasting period resulted in hypoglycemia within 72 hours.

Comment. The hypophysectomized dog can maintain its blood sugar level on a sufficient protein intake. It is evident, however, that even after periods of high protein feeding, its endogenous protein reserves are insufficient to maintain its fasting blood sugar level for very long as compared to the fasting normal dog. If we consider two dogs of equal nutritional status, one normal and the other hypophysectomized, both will have an equal store of available protein to begin with. Upon fasting, the hypophysectomized animal excretes somewhat less nitrogen than the normal animal (4) so that upon any subsequent day of starvation it still has available more body protein than the normal dog. Nevertheless, the protein which it still has available is insufficient to maintain its blood sugar level indefinitely. It becomes obvious that the normal animal must have some other source of carbohydrate besides protein which the hypophysectomized animal lacks. Moreover, the administration of fat to the otherwise starving normal animal will prolong its survival and therefore its total sugar formation during starvation, while the results of feeding fat to hypophysectomized animals do not differ significantly from those of complete starvation. Since the total carbohydrate stores of either animal are too small to affect the results for more than a few days, there remains only fat as the source of the additional carbohydrate in the normal animal. From this point of view, the removal of the hypophysis can be regarded as resulting in the loss of some factor or factors which are responsible for the conversion of fat to carbohydrate. This is in agreement with the observations of many investigators as to the presence of "fat-metabolism," "ketogenic,"

and "diabetogenic" hormones in the anterior lobe of the hypophysis (vide Collip, 11).

THE MODIFIED DIABETES IN THE HYPOPHYSECTOMIZED-DEPANCREATIZED DOG. It is reasonable to assume that the same disturbance which causes a disability of the hypophysectomized animal as regards maintenance of the blood sugar level should be responsible for the ameliorated form of diabetes which has been described in the hypophysectomized-depancreatized animal. The fact that the latter animal, like the hypophysectomized animal with the pancreas intact, becomes hypoglycemic on fasting is hardly susceptible to any other interpretation. Since, as we have shown, the blood sugar level of the hypophysectomized animal is largely dependent upon the diet and the nutritional state, it is to be expected that the degree of diabetes in the hypophysectomized-depancreatized animals should be dependent upon the same factor. This would account for the variability in the diabetic state of the hypophysectomized-depancreatized animals which have been described by various authors.

Since the 1930 report of Houssay and his associates on the hypophysectomized-depancreatized dog, we have studied a number of such animals. In view of the recent nature of such studies, the relatively few confirmatory reports in the American literature, and their important bearing on the question of the fundamental metabolic disturbance in pancreatic diabetes and diabetes mellitus, it seems desirable at this time to briefly summarize our results.

Methods. The seven hypophysectomized-depancreatized animals upon which we are reporting are those, amongst a much larger group, which gave satisfactory evidence of complete hypophysectomy and pancreatectomy by behavior during life, length of survival without insulin, and post-mortem examination.

Following complete recovery from the operative procedure of hypophysectomy, the animals were pancreatectomized. Glucose was administered intravenously until the animals were able to retain food, when they were placed on diets of lean meat, raw pancreas, and cane sugar similar to those used in our previous studies on the depancreatized dog. No insulin was given at any time except in a few isolated instances to demonstrate the sensitivity of these animals towards this hormone.

Results. Figure 3 illustrates an experiment in which a hypophysectomized-depancreatized dog was fed different amounts of lean meat every second day, with a day of fasting intervening between each day upon which food was given. Note the quantitative relationship between the amount of meat given and the initial height and course of the blood sugar curve on the subsequent day of fasting. The rapidity of the onset of the hypoglycemia of fasting, as compared to that which occurred in the hypophysectomized animals (figs. 1 and 2), is also of interest.

The pertinent data as regards the urinary excretion of sugar and nitrogen of our hypophysectomized-depancreatized animals, are summarized in table 1. It may be seen that, in agreement with the work of Houssay and others, our animals survived without insulin for as long as 15 weeks. Their diabetic manifestations on equivalent diets were less than those obtained in depancreatized dogs with the pituitary intact. Like the hyperglycemia (fig. 3) the glycosuria was directly dependent upon the amount of

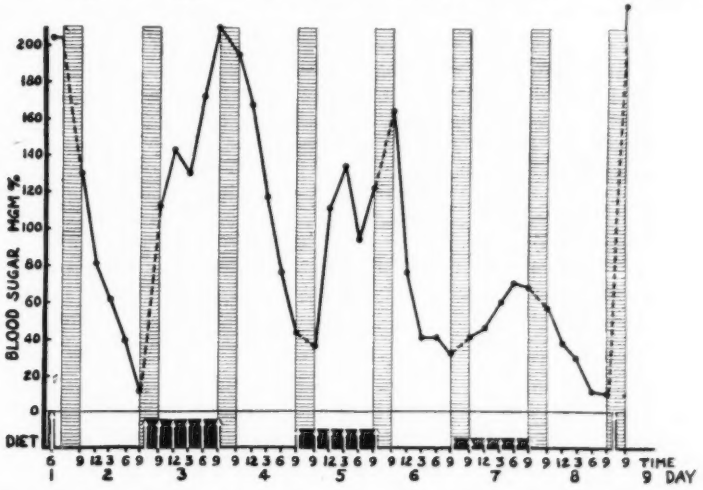

Fig. 3. Influence of the amount of protein intake on the blood sugar level of the hypophysectomized-depancreatized dog. (This is the same animal as depicted in figure 1, before pancreatectomy.) The black areas represent days upon which the animal was fed; the superimposed white arrows indicate the meals. The shaded strips are a foreshortened representation of the night periods, between 9:00 p.m. and 9:00 a.m.

The total amount of food given on the respective days of feeding was as follows: Day 1, 400 grams lean meat, 60 grams cane sugar, 120 grams raw pancreas; day 3, 378 grams protein as lean meat; day 5, 168 grams protein; day 7, 90 grams protein; evening of day 8, same as day 1.

food which the animals accepted. But, regardless of the degree of the diabetic manifestations, no ketonuria was observed.

Comment. Considered from the point of view of the utilization of carbohydrate, our results on the hypophysectomized-depancreatized dogs offer an irreconcilable contradiction. On the one hand, the hyperglycemia and glycosuria following protein and carbohydrate feeding would indicate an inability to utilize carbohydrate; while, on the other hand, the rapid onset of hypoglycemia during fasting would have to be interpreted as an increased rate of sugar utilization. Like the results on the hypophysec-

tomized animal, these data are much more readily interpreted in terms of a disturbance in gluconeogenesis. The diabetic manifestations following protein feeding leave no doubt as to its availability for sugar formation. But the hypoglycemic effect of fasting, in spite of ample fat stores, the low D:N ratios, the lack of ketosis, and the relatively long survival without insulin, are all consistent with the hypothesis that the hypophysectomized-depancreatized animal differs from the depancreatized in that the latter derives sugar from both protein and fat, while the former can use only protein.

TABLE 1

Hypophysectomized-depancreatized dogs

DOG	SURVIVAL (WITHOUT INSULIN)	DIET 400 GM. MEAT; 60 GM. SUGAR; 120 GM. PANCREAS	KETONURIA	AVERAGE GLUCOSE EXCRETION	AVERAGE NITROGEN EXCRETION	AVERAGE D:N ratio
	weeks			*gm./24 hrs.*	*gm./24 hrs.*	
H 7	4	Full	None	10.1	5.1	0
		Partial ($\frac{1}{4}$)	None	2.4	2.9	0
H 11	6	Full	None	80.0	14.3	1.4
		—	—	—	—	—
H 35	7	Full	None	75.0	11.7	1.28
		Partial ($\frac{1}{4}$)	None	6.1	4.9	1.24
H 14	9	Full	None	83.0	15.9	1.50
		Partial ($\frac{1}{4}$)	None	33.5	7.0	0.50
H 30	13	Full	None	70.3	12.0	0.36
		Partial (0)	None	0.6	1.8	0
Sally	14	Full	None	61.8	15.0	0.12
		Partial ($\frac{1}{4}$)	None	39.5	14.0	0
H 4	15	Full	None	95.9	16.5	2.10
		Partial ($\frac{1}{4}$)	None	77.4	12.9	2.50

From this point of view, it is evident that the amelioration of pancreatic diabetes by hypophysectomy is more apparent than real. The essential metabolic disturbance caused by pancreatectomy, i.e., the loss of ability to regulate the rate of gluconeogenesis, is not obviated by hypophysectomy. The latter procedure merely limits the rate of gluconeogenesis by confining the process to protein. This accounts for the marked diabetic manifestations on a high protein intake, and their disappearance during undernutrition. The relatively small protein reserves as compared to fat, even in well-fed animals, accounts for the rapidity of the onset of hypoglycemia

during fasting. It has been noted that the onset of hypoglycemia in the hypophysectomized-depancreatized animal is even more rapid than in the hypophysectomized animal (fig. 3 as compared to figs. 1 and 2). This is in accord with the finding of Biasotti and Houssay (7) on the phlorhizinized hypophysectomized dog and is probably accounted for by the subnormal reserves due to excessive squandering of body protein in uncontrolled sugar formation, consequent to pancreatectomy and phlorhizinization.

Discussion. The metabolic disturbance following hypophysectomy, both in normal and depancreatized dogs, is best explained as being due to an interference with the normal mechanisms of gluconeogenesis from the non-carbohydrate precursors. Our results indicate that while the animal with hypophysis intact derives blood sugar from both protein and fat, the hypophysectomized animal can use only protein for this purpose.

Biasotti and Houssay (7) have attempted to explain similar phenomena on the assumption that hypophysectomized animals have a diminished capacity to form sugar from endogenous protein. We do not feel that this hypothesis adequately accounts for all of the phenomena which we have observed. Their conclusion rests chiefly upon the low D:N ratios obtained in hypophysectomized-depancreatized animals, and depends upon a complete acceptance of the orthodox interpretation of the D:N ratio. We have dealt with the latter question in a previous communication (14) and do not wish to resume that discussion here. It is obvious, however, that if the ordinary values of the D:N ratio depend upon a formation of sugar from fat as well as protein, then the withdrawal of fat from availability by hypophysectomy will decrease the dextrose excretion without affecting the nitrogen and thus lower the D:N ratios obtained.

The amelioration of the diabetic syndrome in depancreatized dogs by hypophysectomy, an operation which interferes with gluconeogenesis, is in accord with the hypothesis that diabetes as it occurs in the depancreatized dog or in diabetes mellitus is due to the overproduction of carbohydrate rather than a failure in utilization. This agrees with the earlier report of one of us (SS, 14) on the utilization of carbohydrate by completely depancreatized dogs not receiving insulin, when maintained on an undernutrition diet composed solely of protein. These animals survived 4 to 6 weeks and became progressively less diabetic the longer they survived. There was a progressive lowering of the D:N ratio, a gradual increase in the respiratory quotient, and an increasing retention of administered sugar, which had a protein-sparing and antiketogenic action. These criteria of "carbohydrate oxidation" became apparent as the fat stores of the animal were depleted. From these results, which were subsequently confirmed by Ring (15), it was concluded that depancreatized animals are able to utilize carbohydrate; that the essential metabolic disturbance is an overproduction of sugar from protein and fat; and that these facts become apparent only when the fat stores of the body have been exhausted.

The hypophysectomized-depancreatized dogs in the present study behaved similarly in all respects to the protein fed, undernourished, depancreatized dogs described above. The differences between the two sets of animals are quantitative rather than qualitative. The animals in the previous study suffered a gradual and incomplete loss of body fat as the period of undernutrition progressed, while the present animals exhibited an acute loss of ability to utilize the ample fat stores which were present.

SUMMARY AND CONCLUSIONS

The hypophysectomized animal differs from the normal in that the latter derives sugar from both protein and fat, while the former is unable to convert the fat and, when its carbohydrate stores are depleted and exogenous carbohydrate is not available, derives its blood sugar from protein alone. This hypothesis accounts for:

1. The hypoglycemic effect of fasting in the hypophysectomized dog.
2. The fact that protein (or carbohydrate) feeding maintains the blood sugar level of the hypophysectomized dog and alleviates the hypoglycemia of fasting, while fat is inert in both respects.
3. The persistence of the hypoglycemic effect of fasting in the hypophysectomized animal even after pancreatectomy.
4. The milder form of diabetes in the adequately fed hypophysectomized-depancreatized animal as compared to the depancreatized animal.
5. The lack of ketonuria without regard to the degree of hyperglycemia and glycosuria in the hypophysectomized-depancreatized animal.

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THE NATURE OF THE T WAVE POTENTIALS IN THE TORTOISE HEART[1]

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As in our previous analysis of the R wave (1) the method employed was to record the action potential at different points in a circular disc conducting field with the heart at the center. Since the theoretical distribution of potential in such a field due to charges near the center is known, the distribution actually obtained around the heart may be compared with this known distribution, and the arrangement of charges in the heart responsible for the action potential may thus be deduced. In the R wave potential analysis, it was found that the electrical changes within the heart which produce the initial ventricular potential complex are the successive growth and decay of two perpendicularly oriented electrical dipoles. The object of the present work was to determine the electrical changes in the ventricle responsible for the T wave deflection.

METHOD AND EXPERIMENTAL PROCEDURE. Reference is to be made to the previous publication (1) concerning the initial ventricular potentials, where the general method is described in detail.

Two series of experiments were performed, the first on hearts well filled with blood, the second on hearts whose blood had been allowed to escape. As in the previous work, an electrogram, obtained by direct leads from the ventricular muscle wall, was recorded simultaneously with the potential curves from different points in the field. This served as a time reference for the field potential curves. Field records were taken at a distance of 10 cm. from the center of the heart, on axes every 15 degrees around the heart. The heart, as before, was placed at the center of the conducting field with its base at 90 degrees and its apex at 270 degrees.

EXPERIMENTAL RESULTS. The T potential deflection, much like the R complex, shows a progressive change in form from monophasic to diphasic and reverse, as the field electrode is moved around the heart. This change, however, is out of phase with the R complex change, and in general, the magnitude, form and duration of the T deflection are much more variable than those of the R. There is a definite progression in time of the wave

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crests along the different axes. The potential curves along opposite axes are mirror images of each other, reflected across the base line.

The records from the two series of experiments, one on hearts full of blood, the other on empty hearts, differed considerably in their characteristics. Two records obtained in an experiment on an empty heart are shown in figure 1. The lower curve of each of the records is that obtained from direct leads to the ventricular muscle wall. The maxima of the R and T deflections of this timing record marked synchronous points for the field potentials recorded in the upper curve. The record made on the 60

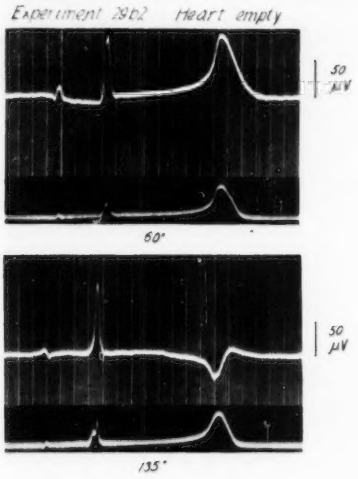

Fig. 1. Two records from empty tortoise heart. Upper curves are records of the field potential at a distance of 10 cm. and along the 60° and 135° axes respectively. The lower curve in each case is the electrogram recorded from direct leads on the ventricular wall. Calibrations in microvolts of the field potential curves are given at the right. The figure is approximately one-quarter the size of the original record.

degree axis shows the characteristically high amplitude and elevated take-off of the T potential in empty hearts. The R and T deflections here are in the same direction, as they are in much the greater part of the field around empty hearts. The record made on the 135 degree axis shows a diphasic T whose chief deflection is opposite to R, as occurs on a few axes.

The tracings of the records made along axes 15 degrees apart from 0 degrees to 180 degrees of two hearts, one full of blood, the other empty, are given in figure 2. These records were chosen as fairly representative of the two series of experiments. The T potential is considerably greater in magnitude in the empty heart. The average maximum T potential was

85 per cent greater in the series of experiments on empty hearts as compared with the series on hearts well filled with blood. The corresponding increase of R potential in the empty as compared with the filled heart was less than 50 per cent. A part of this difference in magnitude of potential in the two series of experiments may be due to differences in resistance in shunting circuits within the heart when the heart is empty and when it is filled with blood. The difference in magnitude of the effect on the R and T potentials shows however that some other factor or factors, at least as regards the latter potential, must be different in the two cases.

ANALYSIS AND DISCUSSION. The distribution of potential during the T complex in the conducting field surrounding the heart necessitates, as in the case of the R complex and that of the action potential of skeletal

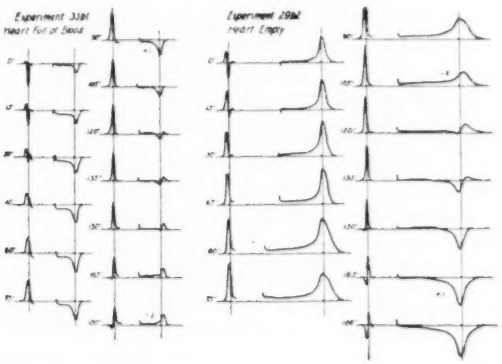

Fig. 2. Tracings from field potential records taken at 10 cm. from the heart and along axes 15° apart. Two experiments, one on an empty heart, the other on a heart well filled with blood. Vertical lines mark synchronous potentials.

muscle, the assumption of electrical dipoles within the muscle as the fundamental cause of the field of potential. It should be emphasized that this assumption, based on the mathematical-physical theory of potential, is unique, in that by no other assumption of charge distribution is it possible to explain the potential distribution observed.

An electrical dipole may be defined as two equal electrical charges of opposite sign a finite distance apart.[2] The axis of the dipole is that of a line connecting the two charges. In an infinite lamina, or in a circular disc, the potential at any point in the field surrounding the dipole is a function of two variables, the distance of the point from the center of the dipole and the angle that a line connecting the point with the center of the

[2] In the current flow case, which is the one here considered, the dipole is maintained, i.e., it consists of a source and sink of equal strength.

dipole makes with the dipole axis. The maximum potential is found along a line representing the extension of the dipole axis. For any point in the field at a certain distance from the center of the dipole, the magnitude of the potential relative to the maximum potential at that distance will be given by the projection of the line connecting the center of the dipole with the point at which the maximum potential is obtained, on the line connecting the center of the dipole with the point in question.[3] This potential is positive or negative depending upon whether the point lies nearer the positive or negative pole of the dipole. Points which lie along a line at right angles to the center of the dipole axis are equidistant from the two charges of the dipole and their potential is zero.

If the dipole is a transitory phenomenon, it has a period of growth and decay, and the potential may be recorded as a potential-time curve from any point in the field. The records thus obtained are all monophasic, except along the line perpendicular to the dipole axis at its center, where the potentials are zero. In the case of a single dipole, its axis, relative magnitude and form of growth and decay are completely determined by the potential-time curves recorded from the field surrounding it.

When the potential-time curves from any part of the field are diphasic or of more complicated form, two or more dipoles are present within the field. In the case of two transitory dipoles, the axis, relative magnitude and form of each can still be determined by taking advantage of the fact that there exist two axes in the field along which one of the dipoles respectively produces no potential, and the potential-time curve along these axes is the result of only one of the dipoles. The curve obtained along this axis perpendicular to the axis of one of the dipoles will give the form of growth and decay of the other dipole. This curve will be monophasic since it is the projection of only one dipole. On one side of this axis the curves obtained will be diphasic, on the other side they will remain monophasic. This is true because the curve obtained on any axis due to the successive growth and decay of two dipoles will be monophasic if the projections of the two dipoles are of the same sign, and diphasic if these projections are of opposite sign. The curve along the axis perpendicular to one of the dipoles will thus represent the boundary between a monophasic and diphasic region of the field. For convenience this curve may be called an index curve.

In a previous work it has been shown that two successive dipoles with asynchronous peaks and on different axes will explain to a close approximation the potential distribution during the R complex. The same has

[3] If V_o is the potential for a point lying on the extension of the dipole axis, the potential at any point, p, in the field lying the same distance from the center of the dipole is $V' = V_o \cos \theta$, where θ is the angle that the line from p to the center of the dipole makes with the dipole axis.

been found to be true in the present work with reference to the T complex. As indicated above, by finding two axes on which transition from monophasic to diphasic curves occur, the form and position of both dipoles can be determined. Thus in figure 2 at 105 degrees in the empty heart record, the first T dipole T_1 is zero, and the record on that axis gives the form of the second T dipole T_2. T_1 begins to add to T_2 in the same direction in the 90 degrees record, and its effect in the opposite direction begins to appear at 120 degrees. Similarly the record at 165 degrees gives the form of T_1. In the full heart record, the 90 degree and 180 degree curves give the form of the two dipoles for the T deflection of that experiment. It may be

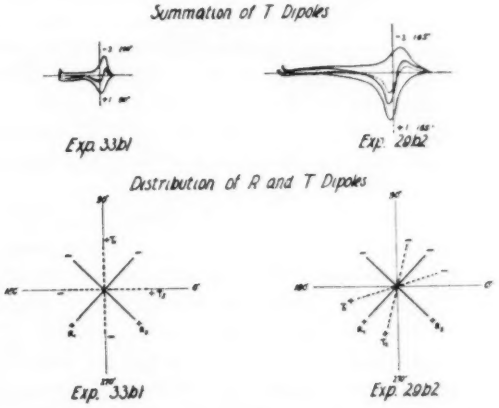

Fig. 3. In the upper half are shown the summations of the T dipole index curves in two experiments. In each case the solid diphasic curve is the resultant of the summation; the broken curves give the tracings of the actual records taken half way between the index axes. The lower half of the figure shows the orientation of the R and T dipoles in these two experiments. The solid lines represent the R dipoles, the broken lines the T dipoles.

noted that the two T dipoles are not quite of the same form. In many of the experiments they differed greatly.

If, then, these index curves give the form of the two T dipoles, and if each is on an axis perpendicular to the axis of the other dipole, they must be symmetrical projections of their respective dipoles, and therefore their summation should give the form of the diphasic record half way between them. Thus in each of these two experiments, if the index curves be added, a curve of the form of the respective 135 degree records should result. In the upper half of figure 3 these summations are given. The solid diphasic curve is in each case the resultant of the addition of the monophasic index curves, while the broken curve is a tracing of the 135

degree record. It can be seen that they are, in each case, of the same form. It is to be noted that since the records were taken 15 degrees apart, the position of the dipole indices cannot be determined more closely than that angle.

The positions of the dipoles, of course, can be determined from the fact that the axis of each index curve was perpendicular to the other dipole. Then the axis perpendicular to index 1 is the axis of dipole T_2, and vice versa. The signs of the dipoles are also determined from the signs of the indices, the sign of each index determining, of course, the sign of the nearer end of its dipole. In this manner the position and sign of the two T dipoles and of the two R dipoles may be determined. They are shown in the lower half of figure 3 for the two types of records.

These dipole distributions are again fairly characteristic of the two types of experiment. The R dipoles are close to 90 degrees apart, the position of their positive poles being at 225 degrees and 315 degrees. Their position does not change when the heart is emptied. These characteristics of the R dipoles are remarkably constant throughout the two groups of experiments.

The T dipoles, however, are extremely variable in position, magnitude, and angular separation. Thus the two T dipoles are greatly increased in magnitude and radically changed in position and angular separation when the ventricle is emptied of blood, while the R dipoles remain constant in position and increase in magnitude much less than the T. The axes of the T dipoles do not coincide with those of the R dipoles, and only occasionally are they nearly oppositely oriented. In the empty hearts, they always lie in nearly the same direction as the R dipoles, giving rise to records in which the R and T deflections are both in the same direction. Their most constant characteristic is that in full hearts one or both of their positive poles may be in the general region of the negative R poles, while in empty hearts, the positive T poles are always in the same semicircle as the positive R poles.

On the basis of the theory of membrane depolarization and repolarization (2) (3) as the source of action currents in general, the field about heart, muscle or nerve would, as a first approximation, be expected to conform to that due to two equal dipoles succeeding each other in time, on the same axis, but opposite in orientation. The first of these would result from the depolarization process, the second from the repolarization process postulated in that theory. A dipole distribution closely approximating this theoretical distribution was reported by us in a recent communication (4) as existing in the frog's gastrocnemius muscle, the only discrepancy being the six- to ten-fold difference in the amount of electricity produced in the two processes.

The dipole distribution found in the present analysis of the action

potential of the tortoise ventricle differs markedly from the simple distribution predicted on the basis of the membrane theory. It is easily conceivable that the two R dipoles may be associated with a split depolarization process proceeding in two asynchronous stages on perpendicular paths. It is somewhat more difficult to understand why the repolarization process, by necessity also split, and thus giving rise to the two T dipoles, does not follow the same two paths. That is, assuming divided depolarization and repolarization processes, the predicted distribution would be that of two R dipoles separated in time and direction, followed by two T dipoles, each on the same axis as its corresponding R dipole, and opposite to it in direction. The situation actually observed differs from this in that the T dipoles do not in general fall on the axes of the R dipoles, and furthermore shift considerably in direction upon altering the pressure in the ventricle by allowing its blood to escape.

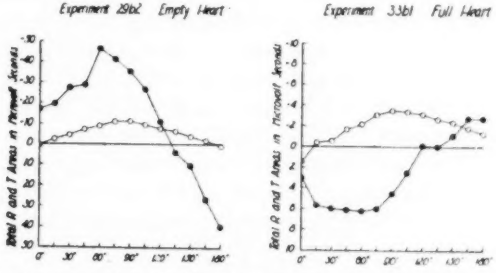

Fig. 4. The total areas under the R and T curves expressed in microvolt seconds. The open circles represent the R areas, the solid circles the T areas.

Another deviation from the theoretical distribution is, as in the case of the gastrocnemius muscle, the inequality of the amounts of electricity produced during the initial and final phases of the ventricular action potential curves. The quantities of electricity produced along various axes during the inscription of both the R and the T potential curves are given for the two types of experiments in figure 4. The diagrams express the quantity of electricity produced in terms of the areas under the potential curves obtained along the various axes around the basal half of the heart. Around the apical half of the heart the curves would be the same except reversed in sign. For the full heart, the quantities of electricity produced during the inscription of the R and T curves over the greater portion of the field are opposite in sign; for the empty heart they are of the same sign. In both cases the R variation of electrical quantity as a function of the angle is out of phase with the T variation. In each case only one axis can be found on which the R and T quantities are equal in magnitude and opposite in sign. The sudden increase in the T quantity in figure

4 for the empty heart between 45 degrees and 60 degrees coincided with a sudden slowing of the heart between the times at which the potential records from these two axes were inscribed; no corresponding marked change can be found in the R quantity curve. The integrated quantities of electricity of each sign would in both cases be much greater for the T than for the R curves. In these two representative experiments, the maximum T quantity for the empty heart is nearly eight times as great as that for the full heart; the maximum R quantity, less than four times as great. Thus the amounts of electricity produced during the inscription of the R and T potential curves along various axes are in general unequal in magnitude, vary in the ratio of their magnitudes with the angle at which the potential is recorded, and may be opposite or the same in sign.

While these marked differences from the characteristics expected on a consideration of the basic concepts of the membrane theory may conceivably be explained as due entirely to differences in order, sequence, and rate of spread of the two processes of depolarization and repolarization (2), it appears more reasonable to assign these discrepancies to the presence of other, additional sources of electricity in the muscle. These additional sources may be related to physical and chemical changes occurring in activity, as was suggested in the case of the frog's gastrocnemius muscle. On the basis of these added sources of tissue electricity, the failure of the T dipoles to coincide in direction with the R dipoles may quite easily be explained, and the effect of pressure changes in shifting the axes of the T dipoles becomes clear. For if such sources give rise to dipoles during the period of activity of the muscle, their effect on the initial muscle dipole or the R dipoles of the heart will be slight, since these occur at the onset of activity, before the gross chemical and physical changes associated with contraction. Their effect, however, on the T dipoles may be very great, since these develop and decay coincidentally with these gross physical and chemical changes. Considering then that the T dipoles actually observed are the summation of repolarization dipoles and these coincident activity dipoles, it can be seen that the direction, magnitude, and form of the curves of development and decay of the latter may be such as to change merely the magnitude and duration of the final phase of the recorded curves, as in skeletal muscle, or may greatly alter the direction of the resultant dipoles also, as in the heart. Since these activity dipoles must be somehow related in direction to the direction of the muscle fibers and of the contraction, it is obvious that their effect on the gastrocnemius muscle potential distribution must be more simple than their effect on that of the heart, because of the much more symmetrical arrangement of the fibers in the former muscle. Finally, it is clear that the shift of the T dipoles in direction upon emptying the heart of its blood may be due to a dependence of the activity dipoles on the pressure relations existing within the ventricular wall at the onset as

well as during the course of contraction. Such a dependence would certainly follow if a major source of the activity dipoles were the phenomenon of streaming potentials.

CONCLUSIONS

The T potential distribution recorded in a conducting field around the tortoise heart accords with that due to the successive growth and decay in the heart of two electrical dipoles separated in direction. These dipoles are extremely variable in position, as contrasted with the constancy in position of the two dipoles accounting for the R potential, and vary in position markedly under experimental conditions which do not alter that of the R dipoles. The quantity of electricity produced during the inscription of the T potential curves is in general greater than that produced during the R, and may be of the same or of opposite sign. The functional relationship between the amounts of electricity and the angle at which the potential is measured is out of phase for the R and T quantities. The T quantity is affected to a greater extent than the R under varying experimental conditions. These findings are not readily interpreted on the basis of the reversible processes of the membrane theory alone. It appears that the assumption of sources of directed potential in addition to that associated with membrane repolarization, coexisting with the latter in the tissue, may very easily account for the various deviations from the distribution of potential predicted on the basis of the membrane theory.

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THE RELATION BETWEEN VISCOSITY OF THE BLOOD AND THE RELATIVE VOLUME OF ERYTHROCYTES (HEMATOCRIT VALUE)

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The relationship between the viscosity of solutions and the volume of substances dissolved or suspended has been subject to considerable investigation, both theoretic and experimental. Kunitz (1) has employed a formula obtained originally from a theoretical development of Einstein, which now has only empirical support. He found that it applied well to a large variety of suspensions. In this formula (1)

$$\eta = \frac{1 + 0.5}{(1 - \phi)^4} \tag{1}$$

η is the relative viscosity of the suspension, that is, the ratio of the absolute viscosity of the suspension to that of the pure solvent, and ϕ is the volume occupied by the dispersed substance expressed as a fraction of the total volume of the solution. According to Hess (2), the relationship between the viscosity of the whole blood and the hematocrit value is hyperbolic and his view can be formulated as in (2)

$$V_{W.B.} = \frac{V_S}{100 - H} \times 100 \tag{2}$$

in which $V_{W.B.}$ is the viscosity of the whole blood, Vs is the viscosity of the serum, and H is the hematocrit value expressed in per cent. Hatchek (3) and others have stated that the relationship is linear.

This study is based on a series of 503 cases, in each of which a simultaneous observation was made of the viscosity of the whole blood, the viscosity of the serum, and the hematocrit value. The hematocrit value was determined in the usual way and the viscosity was measured in relation to water, by using the Hess viscosimeter. The data were analyzed by statistical methods, and formulas were developed which gave the relationship between the variables considered. In addition to the main series of 503 cases, we also studied fifty-eight cases in which there was a diagnosis

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of polycythemia vera, and in which the hematocrit value varied from 30 to 84 per cent. An attempt was made to treat the entire group of data as a continued series, but we were not successful in finding a single curve that agreed well with both series simultaneously. It was therefore decided that it would be safest, so far as curve fitting is concerned, to include only the 503 cases in which there was no blood dyscrasia.

In table 1 is given a summary of the descriptive statistical constants for the latter group of cases. A linear regression formula was fitted to the data to predict the viscosity of whole blood from the hematocrit value. This yielded equation (3) shown graphically in figure 1.

$$V_{W.B.} = 0.978 + 0.098\,H \tag{3}$$

The predictions from the linear equation (3) agree reasonably well with the observed values of the viscosity of whole blood. No hyperbolic char-

TABLE 1

Statistical constants, 503 cases with no blood dyscrasia

	MINIMUM	MAXIMUM	MEAN	STANDARD DEVIATION	CORRELATION	COEFFICIENT
Viscosity, whole blood	2.4	7.4	4.64 ±0.025	0.86	Hematocrit and viscosity whole blood	0.83 ±0.030
Viscosity, serum	1.6	2.6	1.96 ±0.004	0.14	Hematocrit and viscosity serum	0.12 ±0.030
Hematocrit value, per cent	16	58	37.47 ±0.220	7.32	Viscosity whole blood and viscosity serum	0.34 ±0.030

acter in the relationship of the viscosity of whole blood to the hematocrit was noted when the data were studied this way. However, since the values for the viscosity of serum varied in the different cases, and since there is a small, but significant, correlation between these values and the viscosity of whole blood, it was necessary to study the relation as for a constant value of viscosity of serum. This was done by expressing the results of viscosity in terms of relative viscosity, that is, the ratio of the viscosity of the whole blood to the viscosity of a serum. A linear and also an hyperbolic regression were fitted to give relative viscosity from hematocrit value. These regression formulas as determined were

$$\eta = 0.608 + 0.047\ \mathrm{H} \tag{4}$$

$$\eta = \frac{1}{0.793 - 0.00888\ \mathrm{H}} \tag{5}$$

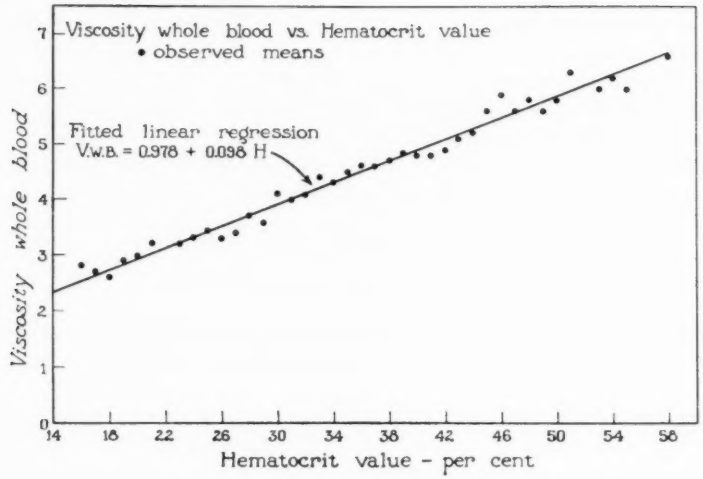

Fig. 1. Viscosity of whole blood and hematocrit values.

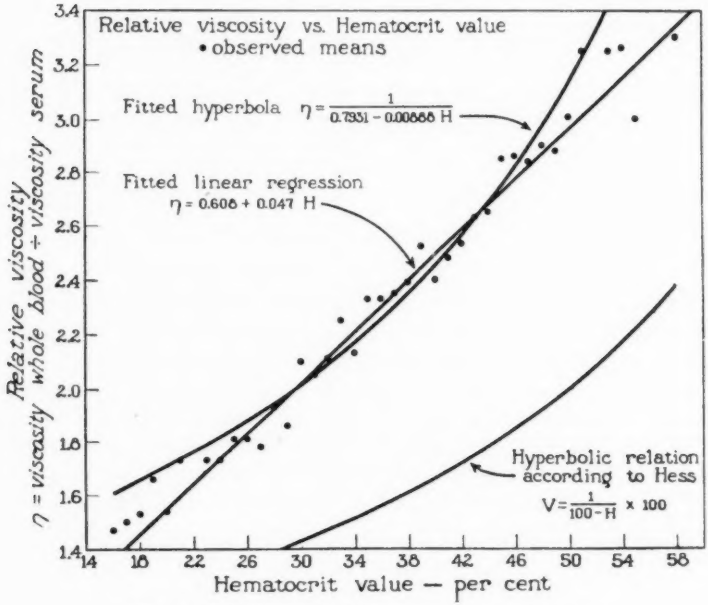

Fig. 2. Relative viscosity and hematocrit values.

in which η is the relative viscosity obtained as the ratio of viscosity of whole blood to viscosity of serum, and H is the hematocrit value in per cent. Neither of these equations or the observations agree with the values of relative viscosity given by formula (1), which was found by Kunitz to agree with observations made on a large variety of suspensions other than blood. The values given by equation (1) for relative viscosity are two to six times as large as our observations, the ratio increasing with increasing hematocrit value. Our fitted hyperbolic equation (5) does not give as good an agreement with the observed means as does the fitted linear equation (4). Neither agrees with the values of the hyperbolic formula of Hess. Our values are consistently and considerably higher than those given by Hess. These several formulas and comparisons are shown graphically in figure 2.

The observations made in the fifty-eight cases of polycythemia vera do not follow the extrapolation of the linear regression given for the cases in which there was no abnormality of the blood. The values of viscosity for large values of hematocrit are higher than would be predicted from the linear formulas given. This would tend to corroborate the idea that for higher hematocrit values than found normally, the relationship of viscosity to hematocrit value becomes hyperbolic. However, our series was too small to make it possible to make a quantitative estimation of this relationship and our equations were therefore confined to the series in which no blood dyscrasia was found.

SUMMARY

The relationship between viscosity of the blood and hematocrit value, the latter in the range of about 15 to 50 per cent, can be well expressed by linear formulas for which equations are given in the text. For values of hematocrit higher than about 60 per cent, found in patients who were suffering from polycythemia vera, the viscosity was higher than the values given by these formulas. If relative viscosity is measured as the ratio of viscosity of the whole blood to viscosity of the serum, the relationship of relative viscosity to hematocrit does not agree with the formula of Einstein. Our observations do not agree with the hyperbolic formulation of Hess.

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THE SYNTHESIS OF NEUTRAL FAT BY THE INTESTINE OF DIABETIC DOGS

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Munk (1) discovered that when fatty acid is introduced into the intestine of a normal animal almost all of it appears in the chyle as neutral fat. Our investigation was undertaken *primarily* to ascertain if the intestine of a depancreatized diabetic dog has the ability to absorb fatty acid and to effect the synthesis of neutral fat. Or, can the intestine in the absence of insulin produce glycerol and combine it with fatty acid? The answer to this question is of special metabolic significance in view of the report of Verzar and Laszt (2) that monoiodoacetate and phlorhizin, which impair phosphorylization in muscle, prevent the absorption of fatty acid.

In addition the lipoid phosphorus and cholesterol content of the chyle was followed in a few of the experiments. This was done because it is reported (3, 4) that the phospholipin and cholesterol content of intestinal lymph increase on neutral fat absorption in rabbits.

METHODS. In our experiments the absorption of the fatty acid from the intestine was studied by collecting and analyzing the lymph from the left thoracic duct.

The normal dogs were fasted for 48 hours prior to the cannulation of the thoracic duct. The depancreatized dogs were given 8 or 10 units of insulin daily for 6 or 8 days after the operation. This dosage of insulin was about one-half that required to keep the urine sugar free. During this period they were fed a diet of fresh raw ground pancreas (200 gm. daily), ground cooked meat and milk. Insulin and food were then withdrawn for 48 hours prior to the cannulation of the thoracic duct, the blood sugar at the time of cannulation being found to be 220 plus milligrams per 100 cc. and the urinary sugar from 4 to 7.5 per cent. The thoracic duct was cannulated under *pento-barbital* (nembutal) anesthesia. A fasting sample of lymph was collected, and then an oleic acid (0.5 cc. per pound body weight) emulsion was introduced into the stomach by means of a tube. The fatty acid emulsion consisted of the following constituents: oleic acid (washed with hot water, 0.5 cc. per pound of animal), 2 grams sodium oleate, 2 grams

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dried ox bile (ether insoluble fraction) and 10 grams of gelatin. The total volume of emulsion introduced into the stomach amounted to about 200 cc. The lymph was then collected for from 6 to 13 hours.

Total fatty acids were determined by the method of Bloor (5), free and total cholesterol by the method of Okey (6), lipoid phosphorus by the method of Kuttner-Lichtenstein (7), and the fat-soluble ester of glycerol by the method of Freeman and Friedemann (8). Duplicate analyses were always made.

RESULTS. The chyle of four normal and eighteen diabetic dogs was studied before and after the introduction of oleic acid into the stomach.

TABLE 1

Showing no appreciable difference between the glycerol content of the chyle of normal and diabetic dogs after administering 0.5 cc. oleic acid per pound body weight enterally

TIME	NORMALS GLYCEROL MGM. PER 100 CC.				DIABETIC* GLYCEROL MGM. PER 100 CC.						
	Dog 1	Dog 2	Dog 3	Dog 4	Dog 1	Dog 2	Dog 3	Dog 5	Dog 6	Dog 7	Dog 8
Control		57	26	24	121	34	70	160	52	42	21
1	234		42		139						
2	90	210	42		231	89	138				
3	117		58	60	176			239	78	63	136
4	125	346	72		222	122	212				
5	135		137		185			185	210	115	220
6	263	239	123	137	214	138	177			125	
7	296		149		237			81	160		226
8	300	226	214	160	222	142					
9	286		240		232				110	76	180
10	358	260	224	184	163		153				
11			97		115	71					
12			99								

* In diabetic dog 4 chyle ceased flowing after 4 hours. Glycerol in initial sample was 58 mgm., in 3rd hour sample 99 mgm.

Of the eighteen diabetic animals only eight showed an increase in fat (table 1) by analysis, or drained milky chyle. In the remaining ten it was found that the stomach failed to evacuate the emulsion. This was evidenced by the fact that post-mortem examination of these dogs, in contrast to the other eight, revealed little or no emulsion in the intestine, and the lacteals were not injected with milky chyle.

The results on both the normal and diabetic dogs are shown in table 1. The results are expressed in milligrams of glycerol per 100 cc. of chyle. The glycerol determined is only that in the form of an ester soluble in fat-solvents, which serves as a true index of the neutral fat present (8). It is evident from the results that the depancreatized diabetic dog can absorb

fatty acid and can also convert the absorbed fatty acid into neutral fat. It does not appear that the synthesis is impaired by the diabetic state, although a statistical study would be necessary to settle this point.

TABLE 2

Showing the data of the analysis of the chyle obtained from normal dog 3 after administering 0.5 cc. of oleic acid per pound body weight enterally

TIME	VOLUME OF LYMPH	GLYCEROL	LIPID P	CHOLESTEROL		TOTAL FATTY ACIDS
				Free	Total	
hours	*cc.*	*mgm. per 100 cc.*	*mgm. per 100 cc.*	*mgm. per 100 cc.*	*mgm. per 100 cc.*	*gm. per 100 cc.*
2 (control)	25	23.8	7.4	18.5	36.5	0.288
3–4	38	60.5	8.7	24.4	63.5	0.692
4–5	25	137.0	11.0	28.2	73.0	1.350
5–6	22	160.0	12.0	28.2	92.0	1.510
7–8	19	184.0	16.0	33.4	136.0	1.690

TABLE 3

Showing data on thoracic duct chyle of diabetic dogs before and after the enteral administration of 0.5 cc. oleic acid per pound

DOG NUMBER	TIME	VOLUME OF LYMPH	GLYCEROL	LIPID P	TOTAL FATTY ACID	CHOLESTEROL	
						Free	Total
	hours	*cc.*	*mgm. per 100 cc.*	*mgm. per 100 cc.*	*gm. per 100 cc.*	*mgm. per 100 cc.*	*mgm. per 100 cc.*
	Control	27	52.5	9.25	0.40	40	120
	3	57	78	11.88	0.66	40	131
1	5	40	210	14.1	0.78	51.6	131
	7	33	160	14.2	1.34	50.0	131
	9	26	110	11.4	0.58	46.3	129
	Control	20	42		0.302		68
	3	47	63		0.510		68
2	5	28	115		0.965		73
	7	28	125		1.060		63
	9	22	76		0.580		68
	Control	15	21	7.4	0.223		75
	3	58	136	11.6	1.072		87
3	5	56	220	14.1	1.910		87
	7	25	226	13.6	1.800		87
	9	18	180	10.9	1.110		73

In table 2 are shown the results of a "complete" analysis in one of the normal dogs. Analogous results were also obtained in normal dog 2. The rise in lipid phosphorus confirms the observations of Süllmann

and Wilbrandt (3), who observed even a greater rise during the absorption of neutral fat in rabbits. The rise in cholesterol confirms the observations of Süllmann and Visscher (4) who observed a similar rise on feeding neutral fat.

The data in table 3 show that a rise in the lipid phosphorus of chyle occurs also on the absorption of fatty acid from the intestine of the diabetic animal; but surprisingly the cholesterol shows little change.

Discussion. According to our knowledge the mode of formation of glycerol has not been demonstrated in the animal organism. It is apparent from our results that glycerol is readily available in the intestinal mucosa of the diabetic dog for the synthesis of neutral fat. While these experiments cannot be interpreted with certainty as proving that the diabetic animal can synthesize glycerol, we believe such a conclusion to be the most reasonable interpretation of the data.

According to the results of Verzar and Laszt (2), the absorption of fatty acid and neutral fat is prevented in the rat by monoiodoacetate and phlorhizin. If this is true phlorhizin and pancreatic diabetes differ in respect to fatty acid absorption, since the diabetic dog certainly possesses enzyme systems adequate for the absorption of fatty acid. If a phosphorylization process is necessary for the absorption of fatty acids, it is not definitely impaired in extirpation diabetes.

We have no adequate explanation to offer for the failure to find a comparable increase in the cholesterol content of intestinal lymph during the absorption of fatty acid in the normal and diabetic animals. The source of the cholesterol which causes the rise in normal dogs is unknown. It may come either from the intestinal cells or the lumen of the intestine. Since it is claimed by Dam and Starup (9) that esterification of sterols is necessary for their absorption, the absence of the rise may be due either to the absence of pancreatic secretion or to some metabolic defect in the cells of the mucosa due to the absence of insulin. The liver cannot be entirely ruled out (10), although it should be remembered that our dogs received insulin and raw pancreas, which prevents evident liver damage, until 48 hours prior to the experiment. The alteration of cholesterol observed, however, falls in line with the alteration of cholesterol metabolism that is generally recognized to occur in diabetes.

CONCLUSIONS

1. As in normal dogs, oleic acid is absorbed from the intestine of depancreatized diabetic dogs largely as neutral fat and phospholipin. Thus, glycerol is freely available for neutral fat synthesis in the absence of insulin. Neither is phosphorylization of the absorbed fatty acid, as indicated by the rise in lipid phosphorus in the chyle, impaired.

2. During the absorption of fatty acid, the free and total cholesterol

content of the chyle of normal dogs shows an increase which parallels that of the other fatty fractions. This increase is largely absent in the diabetic (depancreatized) dog.

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THE RATE OF ELIMINATION OF DISSOLVED NITROGEN IN MAN IN RELATION TO THE FAT AND WATER CONTENT OF THE BODY[1]

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Quantitative studies of the dissolved nitrogen in man have been previously made only for short periods of time, chiefly in determinations of cardiac output (Bornstein, 1919). Measurements of nitrogen absorption and elimination not only give an approximation of cardiac output, but also afford a representation of the absorption and elimination of all chemically inert gases, specifically the inhalation anesthetics. In connection with the decompression of divers studies of the dissolved nitrogen are of immediate practical importance.

This paper presents the application of certain principles from studies of the dissolved nitrogen in dogs (Shaw, Behnke, Messer, Thomson and Motley, 1935) to measurements of nitrogen in man.

METHOD. Inhalation of pure oxygen results in elimination of the gaseous nitrogen dissolved in the tissues of the body. Oxygen can be breathed for a period of 4 hours in a closed system consisting of a helmet, cooling coil, spirometer, and soda lime cannister. Analysis of periodic samples of the circulating oxygen supplies the data for calculations of nitrogen elimination. The volume of the system was 40 liters so that the oxygen percentage at the end of an experiment did not fall below 96 per cent. The term "nitrogen" is applied to the residual gas in the Van Slyke apparatus after oxygen absorption with hyposulphite.

The nitrogen measurements were made on 3 healthy men of medium build who interrupted their usual activities to breathe oxygen at weekly intervals while resting on a cot.

RESULTS. Figure 1-A is typical of the results from 25 experiments on the 3 men. The solid line represents the actual measurements of nitrogen elimination over a period of 4 hours on subject A. The broken line, drawn from calculations based on the experimental results, represents the nitrogen eliminated during the first 5 minutes (when oxygen replaced the air in the lungs and in the apparatus) and during the period following

[1] This research was aided by the Miriam Smith Rand Fund.

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the fourth hour. Since these calculations indicate clearly the manner in which nitrogen is eliminated, they will be given in detail.

The elimination or absorption of nitrogen with changes in pulmonary nitrogen tension is a function of the circulatory rate and can be represented by one or more exponential equations of the form,

$$(1) \qquad Y = A(1 - e^{-kt})$$

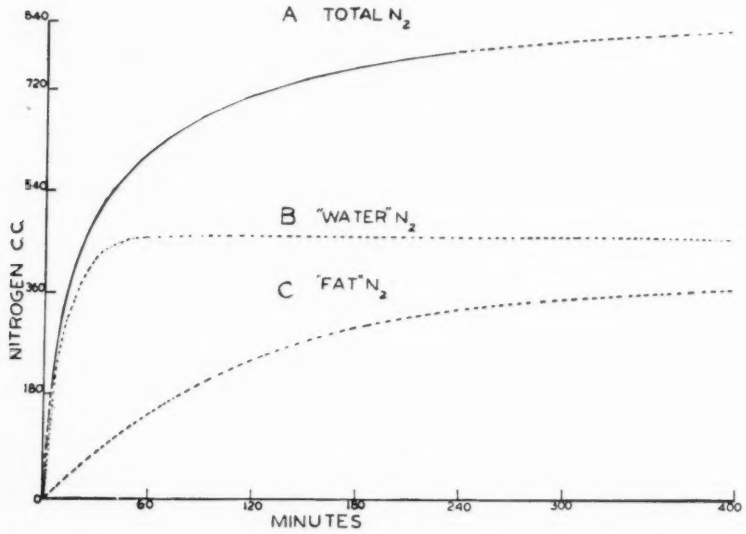

Fig. 1. *Curve A*, nitrogen elimination or absorption of a young, well developed man weighing 60 kgm. Solid line drawn from experimental values, broken line drawn from calculations based on experimental values. *Curve B*, nitrogen elimination or absorption of the water and 16 per cent of the fat and lipoids in the body. *Curve C*, nitrogen elimination or absorption of 83 per cent of the body fat and lipoids.

expressing the relationship that the nitrogen is eliminated from the body at a rate which is a constant percentage of the amount present at any given time. Y represents the value for nitrogen eliminated during the time interval, t; A, the total nitrogen; k, the rate of change in the slope of the curve; and e, the natural base of logarithms. The expression $1 - e^{-kt}$ gives the percentage decrease of the total nitrogen during the time interval, t.

In terms of k, equation *1* becomes

$$(2) \qquad k = \log_e \frac{A}{A - Y} \cdot \frac{1}{t}$$

The value of A in terms of Y can be expressed as

$$A = \frac{(Y_1)^2}{2Y_1 - Y_2} \tag{3}$$

provided that the time, t_2, corresponding to the value for Y_2 is twice that of t_1 corresponding to the value for Y_1. In subject A, for example, the average experimental value for the 5 minutes following the rinsing period of 5 minutes was 116 cc.; and for the 10 minutes following the rinsing period, 187 cc. (table 1, columns 4 and 5). Substituting these values in equation

TABLE 1

Nitrogen elimination during the first 5 minutes (rinsing period) and from the 5th to the 25th minute

SUBJECT	WEIGHT	HEIGHT	AGE	SURFACE AREA	NITROGEN ELIMINATION							CARDIAC OUTPUT	
					Calculated			Experimental				Estimated from N_2 output	Predicted from surface area
					1st min.	Total 1st 5 min.	5-25 min.	Exp. no.	5-10 min.	5-15 min.	5-25 min		
					(1)	(2)	(3)		(4)	(5)	(6)	(7)	(8)
	lbs.	*inches*	*years*	*sq.m.*	*cc.*	*cc.*	*cc.*		*cc.*	*cc.*	*cc.*	*liter*	*liter*
A	132	63	32	1.62	45.5	188	257	A-1	95	165	259	4.7	3.6 ± 0.5
								A-2	124	188			
								A-3	130	208	293		
								Av.	116	187	276		
B	124	63	30	1.57	33.0	143	233	B-1	93	158	248	3.33	3.5 ± 0.5
								B-2	97	160	253		
								Av.	95	159	251		
C	143	68	33	1.77	38.0	165	269	C-1	100	179	298	4.0	3.9 ± 0.5
								C-2	111	184	278		
								C-3	119	189	271		
								Av.	110	184	282		

3, $A = 299$. With this value for A, the rate of change per minute in the slope of the curve, k, can be calculated from equation *2* as 0.098. With values for A and k, the nitrogen eliminated during the rinsing period of 5 minutes (Y) can be obtained from equation *1*. Since the experimental curve is extrapolated 5 minutes to the left of the point of origin at which actual measurements of nitrogen began, t has a minus sign, or,

$$Y = 299\ (1 - e^{-(0.098 \cdot (-) t)}) = 299\ (1 - e^{-(0.098 \cdot (-) 5)})$$
$$= 299\ (1 - e^{0.49}) = (-)\ 188 \text{ cc.}$$

Using the same procedure, the calculated value for the first minute of oxygen breathing is 45.5 cc. Dividing this value by 0.96, the solubility coefficient of blood in vitro equilibrated with air (nitrogen tension did not exceed 580 mm.) at body temperature (Van Slyke, Dillon and Margaria, 1934), and multiplying by 100 gives 4700 cc., a value which is an approximation of the cardiac output during the first minute. The cardiac output of healthy individuals in the basal state can be predicted according to Grollman (1932) as 2.2 ± 0.3 liters per square meter of body surface, giving in the case of subject A 3.6 ± 0.5 liters. The value of 45.5 for the nitrogen eliminated during the first minute of oxygen breathing is, therefore, probably correct within the limits of experimental error. The calculated values for nitrogen elimination during each minute up to the sixth are given in table 1, column 1. The measurements of Campbell and Hill (1931) of the nitrogen elimination during the first 5 minutes corroborate the values in column 2, table 1.

A check on the method of calculation is obtained by extrapolating the experimental curve to the right and comparing the predicted value of nitrogen elimination for 20 minutes (column 3, table 1) with the experimental values, column 6. The calculation, $Y = 299\ (1 - e^{-(0.098 \cdot 20)})$, gives a value of 257 cc. In 6 experiments on subject A, the average of the values for 20 minutes was 265 cc.

It should be pointed out that calculations of nitrogen elimination based on the values for 5 and 10 minutes hold good only for periods up to 25 minutes. If the rate of change, k, in the slope of the nitrogen elimination curve for the whole body were constant, then the value for A would represent the total body nitrogen, and accurate calculations could be made for the whole period of nitrogen elimination. The value for k, however, decreases as a result of unequal blood flow in relation to the nitrogen content of the tissues. At the start of oxygen breathing the average nitrogen tension in the blood is equal to the nitrogen tension in the different tissues of the body, and a maximum load of nitrogen is eliminated per unit of time. As the experiment progresses, the average nitrogen tension of the blood falls below the nitrogen tension in the slowly desaturating or fatty tissues. Consequently the percentage rate of nitrogen elimination decreases. The A value, based on the quantities of nitrogen eliminated at 5 and 10 minutes, more closely approximates the nitrogen in the body fluids, and does not include the large reservoir of nitrogen in the fat which is given up very slowly. More representative values for the total body nitrogen can be obtained by using the figures for nitrogen eliminated in 1 and 2 hours, or 1.5 and 3 hours. The accuracy of the extrapolated values is usually limited to a period of time corresponding to that of the experimental values used in the calculations.

The curve for total nitrogen, curve A, figure 1, can be conveniently rep-

resented by two exponential equations on the basis of a high value for k during the first hour and a lower value for k after the first hour. If that portion of the curve after the first hour be extrapolated to the left on the basis of its k value, then curve C, figure 1, can be drawn. The difference between the nitrogen values for curve C and those of curve A are represented by curve B. Curve A is thus the sum of two components, B and C, or

$$Y = \text{(B) } 458\ (1 - e^{-0.098t}) + \text{(C) } 382\ (1 - e^{-0.0085t})$$

where Y is the total nitrogen eliminated from the body during time, t.

The designations "*fat*" and "*water*" to curves B and C follow from the relation of nitrogen to its solvents. Since fat contains between 5 and 6 times more nitrogen than water (Vernon, 1907) and since the capacity of the blood to carry nitrogen is about the same as water, nitrogen removal from fat and lipoids will take 5 to 6 times longer than nitrogen removal from body fluids. It is a reasonable assumption, then, that the original nitrogen in the body fluids is eliminated at the end of the first hour according to curve B, figure 1.

The nitrogen solvents of the body. While the division of nitrogen in relation to its solvents is given approximately by curves B and C, figure 1, a more exact division follows from the quantitative analysis for water and fat in a dog used in the experiments of Shaw et al. (1935). The water was removed from the tissues of dog D by careful drying after preliminary treatment with 95 per cent alcohol. The fat and lipoids were extracted with carbon tetrachloride. The results: weight of dog, 12.234 kgm.; weight of fat, 1.889 kgm. (15.43 per cent); weight of dry solids, 3.117 kgm.; weight of water by difference, 7.228 kgm. The solubility of nitrogen in omental dog fat, 38°C., 570 mm., was found to be 5.57 cc. per 100 grams—a value in agreement with the extensive studies of Campbell and Hill (1931). The solubility of nitrogen in water, 38°C., corrected to 570 mm., is 0.954 volume per cent (Van Slyke, Dillon and Margaria, 1934). Multiplying the solubility values by the weights of fat and water in the dog:

7.228×9.54 = 69 cc. of nitrogen dissolved in the water.
1.889×55.7 = 105 cc. of nitrogen dissolved in the fat.
Total nitrogen = 174 cc. or 14.2 cc. per kilogram.

The measurements of the nitrogen content of dog D, including the calculated value for nitrogen eliminated during the first 7 minutes (rinsing period), totalled 167 cc. With the exception of a small amount of nitrogen dissolved in hemoglobin, the nitrogen solvents of the body are water and fat.

The total nitrogen content of subject A divided by the body weight, 60

kgm., gives 14 cc. of nitrogen per kilogram, or the same value as that for dog D. Further, the time required for nitrogen elimination in man, 98 per cent in 6 hours, is twice the time required for the dog, and corresponds in the resting state to the ratio of the cardiac output per kilogram of dog to the output per kilogram of man. If the water content of the body is 65 ± 5 per cent of the total weight and if 70 per cent (the usual figure in metabolic studies (Lavietes, D'Esopo and Harrison, 1935)) is used, then the product of the weight of the water, 42 kgm., and the solubility of nitrogen in water, 9.5 cc. per kilogram, gives a value of 400 cc. The nitrogen in the fat and lipoids is 440 cc. (840–400). Dividing 440 by 55.7 gives an estimation of the amount of fat, 7.9 kgm. or 13.2 per cent of the body weight.

The fat and lipoid content of the brain and spinal cord. The relatively high fat and lipoid content of the spinal cord in comparison with the brain is undoubtedly partly responsible for the susceptibility of this tissue to injury from nitrogen bubble formation in compressed air illness. In an extraction of the fat and lipoid substances from the brains and spinal cords of 5 dogs with carbon tetrachloride, 100 grams of brain substance contained 4.8 grams of fatty material, while 100 grams of spinal cord tissue contained 27.8 grams of fatty material. The quantity of nitrogen absorbed by the spinal cord per unit weight is, therefore, about 2.5 times that absorbed by the brain. This fact, in addition to the poorer blood supply, accounts for the more rapid injury of the cord from nitrogen bubble formation compared with the brain.

DISCUSSION AND APPLICATIONS OF THE EXPERIMENTAL RESULTS. *Determination of cardiac output.* The experimental results in this paper do not permit any conclusions with reference to the accuracy of Bornstein's nitrogen elimination method for the determination of cardiac output. They suggest, however, the advisability of further study of this comparatively simple method either in the original or in a modified form. The main objections specifically applicable to the method, listed in the careful study of Marshall, Harrop and Grollman (1928), were essentially removed by their own experiments or by the work of subsequent investigators. The observation that constant values for nitrogen elimination cannot be obtained on untrained individuals may not necessarily invalidate the method.

The absorption and elimination of inhalation anesthetics. The inhalation anesthetics are generally regarded as chemically inert, and consequently an index of their absorption and elimination will be given by the nitrogen elimination curves, A, B, and C, figure 1. For example, the time required for the body to come into equilibrium with a given tension of ether is represented by figure 1, curve A, since the ratio for ether, $\frac{\text{solubility in fat}}{\text{solubility in water}}$, is

4.3—about three-quarters that of the corresponding ratio for nitrogen. If ether elimination follows the nitrogen curve, then about 6 hours will be required for complete elimination following withdrawal of the anesthetic.

The absorption and elimination of anesthetics only slightly soluble in lipoids should follow curve B, while the elimination of members of the aliphatic group will be proportional to their solubility in lipoids.

The decompression of divers. The application of the experimental results to the problems of deep sea diving will serve to emphasize certain fundamental concepts.

The nitrogen elimination experiments on dogs (Shaw et al., 1935) supported three accepted generalizations, although the significance of the third is not usually realized. The principles may be briefly restated: *a*, with the same pressure head the rate of nitrogen absorption (saturation) is equal to the rate of nitrogen elimination (desaturation); *b*, nitrogen absorption obeys Henry's law; and *c*, the time required for complete desaturation of the body is independent of the quantity of nitrogen absorbed, i.e., the same equation with the same constants applies to all experiments irrespective of the amount of nitrogen diffusing.

If these principles are applicable to nitrogen absorption and elimination in man, curve A, figure 1, represents not only the nitrogen leaving the body when the partial pressure of nitrogen is lowered, but also the nitrogen absorbed when the partial pressure of nitrogen is raised. Curves B and C, figure 1, indicate the uptake of nitrogen at increased pressures by the body fluids and lipoids respectively.

The importance of the third principle and the understanding of its implications require further discussion. If, for example, the barometric pressure is raised to 2 atmospheres, the nitrogen absorbed after successive exposures of 30, 60 or 360 minutes will not only be eliminated in the same period of time but also at the same percentage rate when the barometric pressure is again lowered to 1 atmosphere. These statements indicate that the nitrogen solvents, fat, lipoids and water, are so distributed that on decompression after partial saturation the diffusion of nitrogen from the rapidly saturating body fluids into the slowly saturating lipoids and fat tends to equalize the partial pressure of nitrogen in the different tissues of the body. Thus, when the body is only in partial equilibrium with an increased nitrogen tension the lipoids and fat act as nitrogen absorbents when the pressure is lowered, and serve as buffers against bubble formation in the blood stream. Fat men with adequate circulations are, therefore, better suited for short exposures to excess pressure than lean men.

If the results from the dog experiments are applied further, figure 2-A (from fig. 1-A, subject A) will represent nitrogen elimination after an indefinite exposure (e.g., 99 per cent saturation) to any given excess pressure (e.g., 4 atmospheres), while curve B represents nitrogen elimination

after a 30 minute exposure (62 per cent saturation) to the same excess pressure. Thus the nitrogen elimination curve after partial saturation at a high pressure (62 per cent at 4 atmospheres) is indistinguishable from the nitrogen elimination curve after complete saturation at 2.48 ($=0.62 \times 4$) atmospheres. Actually some tissues with a high fat and lipoid content, after partial saturation at a high pressure may not be completely saturated with nitrogen when the pressure is lowered to 1 atmosphere. For it is probable that the bone marrow, consisting largely of fat (as high as 95 per

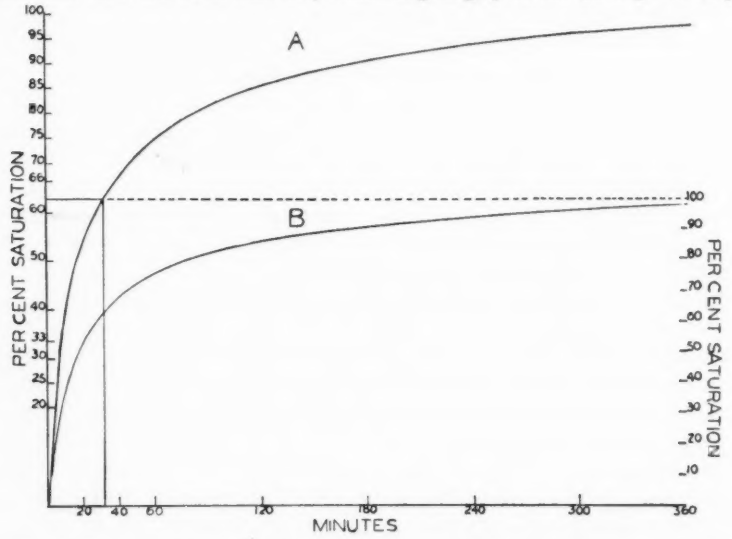

Fig. 2. *Curve A*, reproduction of curve A, figure 1, indicates the percentage saturation of the body as a whole after an exposure to excess pressure for 30 minutes. The product of the pressure in atmospheres, the percentage saturation, and the nitrogen content of the body at 1 atmosphere gives the quantity of nitrogen absorbed. *Curve B* indicates the elimination of the nitrogen (absorbed in 30 minutes according to curve A) when the pressure is dropped to 1 atmosphere, based on the dog experiments of Shaw et al. (1935).

cent) encased in bone, and dependent for its nitrogen absorption on a relatively poor blood supply (Campbell and Hill, 1933), requires many hours for complete saturation. The spinal cord may also saturate slowly because of a high fat and lipoid content (28 per cent).

Duration of exposure to excess pressure with immediate decompression to 1 atmosphere. It follows as a corollary that the same curve will represent the elimination of a given quantity of nitrogen irrespective of whether the nitrogen is absorbed at a low pressure over a long period of time or at a high pressure for a short period of time. If use is made of the empirical

fact that compressed air illness (excessive nitrogen bubble formation) rarely occurs with immediate decompression, even after prolonged exposures to excess pressures, from 1.3 atmospheres (Haldane, 1922), or from even somewhat higher pressures (Japp, 1909), then the safe exposure time to excess pressures for subject A followed by rapid decompression (2 min.) can be predicted from his nitrogen elimination curve (curve A, fig. 1). The calculations are easily computed by making the product of the excess

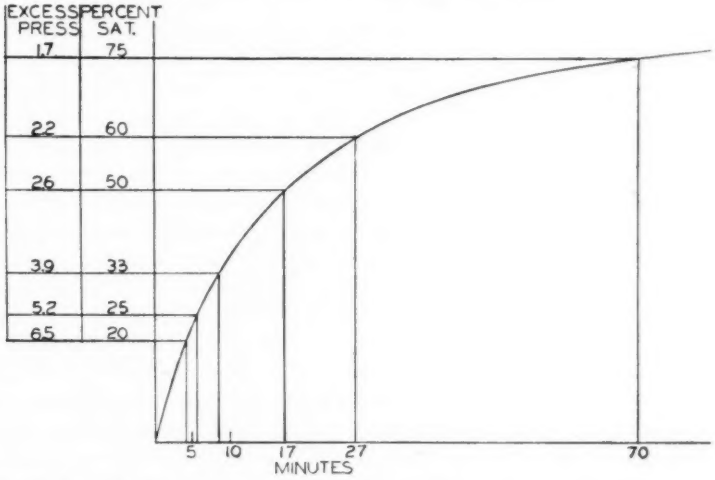

Fig. 3. Duration of exposure to excess pressure followed by immediate decompression to 1 atmosphere, based on curve A, figures 1 and 2, and on the assumption that rapid decompression from +1.3 atmospheres (with the nitrogen tension of the body in equilibrium, i.e., 100 per cent saturation) is safe.

pressure, P, and the per cent saturation of the body, R, equal to a constant, K, which has a value which lies between 1.3 and 1.5. For example,

$$P \times R = K$$
$$1.3 \times 100 \text{ per cent} = 1.3$$
$$2.6 \times 50 \text{ per cent} = 1.3$$
$$3.9 \times 33.3 \text{ per cent} = 1.3$$

The time for any degree of saturation, R, is given by the nitrogen elimination curve. The method of computation is illustrated by figure 3. The results of these calculations have been verified by repeated diving tests (Kagiyama, 1934).

SUMMARY

The inhalation of oxygen results in the elimination of dissolved body nitrogen in equilibrium with pulmonary nitrogen.

The nitrogen content of a young, well developed man weighing 60 kgm. is 840 cc., or 14 cc. per kilogram, 98 per cent of which is eliminated with oxygen breathing over a period of 6 hours.

If the assumption is made that the body is 70 per cent water, then 400 cc. of nitrogen are dissolved in water and 440 cc. in the body fat and lipoids. Dividing 440 by the solubility coefficient of nitrogen in fat gives an estimate of 13.2 per cent for the body fat, in contrast with 70 per cent assumed for body water content.

The fat from a well nourished dog of 12.2 kgm., extracted with carbon tetrachloride, comprised 15.4 per cent of the body weight and the water 59.2 per cent. If these values are multiplied by the respective solubility values of nitrogen in fat and water, the nitrogen content of the dog per kilogram is 14.2 cc., or approximately the same as that for man.

Nitrogen elimination follows an exponential type of curve, the slope of which is a function of the cardiac output. The cardiac output in liters can be estimated by dividing the value for nitrogen eliminated during the first minute by the quantity of nitrogen dissolved per liter of blood.

The rate of absorption and the time of elimination of inhalation anesthetics can be estimated from the nitrogen elimination curve on the basis of the ratio $\frac{\text{solubility in fat}}{\text{solubility in water}}$.

During the decompression of divers who have been exposed for short periods (20 min.) to excess pressures, the fat and lipoids of the body act as nitrogen absorbents and serve as buffers against bubble formation. Under these conditions rapid decompression from relatively high pressures can be safely effected.

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A FURTHER STUDY OF THE ELECTRIC RESPONSES OF SMOOTH MUSCLE

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In an earlier publication (Rosenblueth, Leese and Lambert, 1933), action potentials from the smooth muscle of the nictitating membrane, trigonum of the urinary bladder, pilomotors of the tail and pregnant uterus of the cat were described. These were classified as "initial" and "delayed" potentials, the "initial" being correlated with the excitation of the muscle and the "delayed" with the contractile phase.

In these earlier experiments, the potentials were recorded with a two-stage amplifier coupled by a condenser with a relatively short time-constant. With the acquisition of a new direct-coupled amplifier (Garceau and Forbes, 1934) which was designed especially for the recording of slow potentials of long duration, it seemed of interest to explore more carefully the time relations of the various phases of the electric responses of smooth muscle.

The potentials recorded in the following experiments were obtained from the smooth muscle of the nictitating membrane, activated by stimulation of the cervical sympathetic nerve, from the fundus of the urinary bladder with stimulation of the pelvic nerve (thus including a preparation from the parasympathetic system) and the pilomotors of the tail activated by stimulation of the abdominal sympathetic chains.

METHOD. Cats under dial anesthesia (0.65 cc. per kgm.) were used. The method followed for each of the three different preparations is given in the respective sections.

The stimuli were induction shocks from paired coreless Harvard induction coils. The stimulating electrodes were silver wires set about 6 mm. apart in small-bore rubber tubing. Maximal stimuli were generally used.

The leads for recording the electrogram were either partially insulated silver needles, chlorided at the tips, or silver-silver chloride, agar-saline wicks, which were used to eliminate polarization effects and artefacts dependent on contact changes (cf. Controls). The grid lead was in all cases placed on the active tissue, and the ground lead on indifferent tissue.

The Hindle string galvanometer and the camera described by Forbes,

Davis and Lambert (1930) were employed for recording the amplified electric response. A slack string was used (500 m/amp.).

The myogram was recorded by means of a light aluminum heart lever which was made practically isometric by attaching the muscle close to the fulcrum on the side opposite to the recording arm and making it pull against a rubber band. The lever was placed near the camera in the beam of light from the galvanometer.

For polarizing the tissue in the control experiments, several dry cells, usually 3 to 5, were connected in series with 600,000ω, the wick electrodes and the tissue. The resistance of the leads in agar-saline solution was 3,000ω; that of the tissue, usually 7,000ω. The two leads were also connected by a 200,000ω resistance in the amplifier, which was thus in parallel with the tissue. The amount of current was varied by changing the number of dry cells and the resistance in series with the tissue. The polarity was changed by means of a reversing key. The degree of polarization of the tissue, which was of the order of millivolts, was indicated by a meter in the output of the amplifier.

RESULTS. *I. Nictitating membrane.* Stimulating electrodes were placed on the cervical sympathetic nerve approximately at the level of the thyroid cartilage. For recording, agar-wick electrodes were used. One, the grid lead, was placed on the nictitating membrane, and the other on the connective tissue lateral to the eye. The membrane was in some cases tied to a lever for myographic recording and in others merely held by threads in a stretched position to eliminate artefacts caused by gross contact changes of the leads during contraction (cf. Controls). In the majority of cases, single shocks were used.

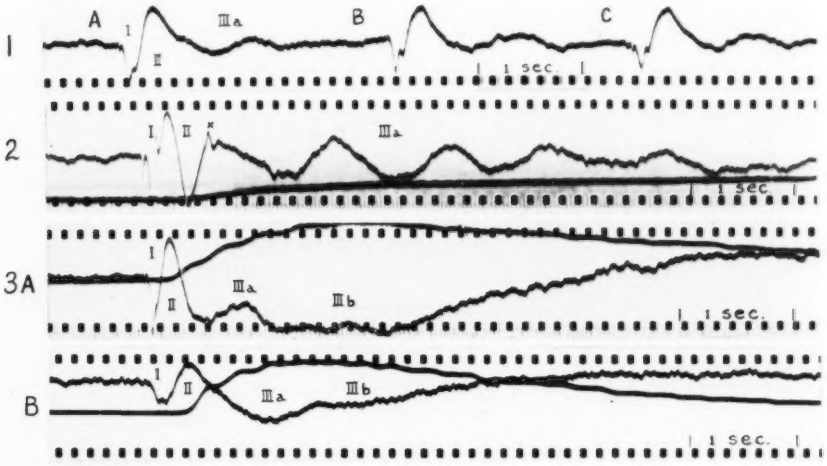
A clearly reproducible sequence of potentials was observed in response to a single shock, as is illustrated in figures 1 to 3. Since the potentials obtained from this preparation will be used as a standard to classify those of the pilomotors of the tail and of the urinary bladder, they will be described in detail.

After a latency of about 40σ there is an initial, or "spike," potential of relatively short duration—about 80 to 100σ (fig. 1). This is almost always monophasic and will be called "I." It is followed in the majority of cases by a slight pause, or period of equipotentiality, of 30 to 40σ in duration. Then follows a larger and more prolonged potential, "II," which is usually of the opposite sign. This may be either monophasic or diphasic (cf. figs. 1 and 2) and lasts for approximately 400σ. Its latency from the moment of stimulation is approximately 160σ.

Following this potential there is sometimes again a slight pause, after which there occurs a third potential, "III." This may take on one of two different forms and may be a composite of both. The first type of wave, "IIIa," is relatively shorter than "IIIb," though slightly longer than II,

and may be monophasic (fig. 3B), diphasic (fig. 1A), or polyphasic and rhythmic (fig. 2). The second type, IIIb, is a much more prolonged potential, lasting throughout the period of contraction and returning to the baseline during or at the end of the relaxation phase (cf. figs. 3A and 3B). In these figures, IIIa is superimposed on IIIb.

Figures 2, 3A and 3B show also the isometric myogram of the contraction. Figure 3 (A and B) indicates that the onset of contraction is correlated with the middle of potential II. In none of the records obtained with the



Figs. 1, 2 and 3. Electrical responses of the nictitating membrane to single make and break shocks applied to the cervical sympathetic nerve.

In these and other records, the stimulus is marked by a sharp artefact in the string record or by an arrow. The numerals I, II, IIIa and IIIb indicate the corresponding potentials (cf. text). The units of the time scale are 40σ. The rapid vibrations in the base line in several records are artefacts due to inadequate shielding of the timer. The figures are reduced one-half; the voltage (microvolts) per millimeter excursion is therefore given for the reduced record.

Fig. 1. A, B and C. A series of responses to single shocks applied at $2\frac{1}{2}$ second intervals. I and II are easily distinguished; IIIa is diphasic. μv./mm. = 14.6. August 2, 1934.

Fig. 2. A response to a single make shock showing a diphasic II and a polyphasic IIIa. A myogram accompanies the electrogram. The small potential, x, is unique and probably of extraneous origin. μv./mm. = 11.2.

Fig. 3. A. A response to a single break shock showing a typical IIIb following the course of contraction. II and IIIa are monophasic.

B. Same. The combination of I and the pause before II make a square-topped wave (cf. text). IIIa is clearly defined and superimposed on IIIb. μv./mm. = 16.6. August 9, 1934.

lever described above (cf. Method), does the beginning of contraction coincide with the beginning of II. The latencies of contraction from the moment of stimulation in figures 2, 3A and 3B are approximately 340, 220, and 360σ, respectively.

The variations from the typical responses to a single shock are slight in the nictitating membrane. The spike potential I is almost always monophasic and usually opposite in sign to that of II. Occasionally it may be reversed and, being then of the same sign as II, can be recognized only by its latency or by a dip separating the peaks of the two waves. Or, again, II may begin so early in the falling phase of the initial potential that a square-shaped wave results as in figure 3B. In a few cases I may be completely reduced through some condition of recording and leave only II.

II may be monophasic or diphasic, the diphasic wave having approximately the same total duration as the monophasic wave. If one considers that the pauses or periods of equipotentiality are produced by the insertion of small potentials between the dominant phases, II may be considered more complex. The sharpness of the break in the falling phase of I seems to indicate that the first pause belongs to II. In figure 1A, the second pause is clearly shown; in figures 1B, 1C and 3B it is revealed by the change in the slope of the falling phase of II. The abruptness of this change suggests that the second pause probably belongs to IIIa rather than to II.

The variations in III have already been noted. IIIb is not always opposite in sign to the first phase of II as in figure 3B, but may be of the same sign.

II. Bladder. The pelvic nerves to the bladder were cut on the two sides and one or both placed upon the stimulating electrodes described above. The hypogastric nerves were also cut, so that the bladder was completely denervated. In some cases they too were stimulated.

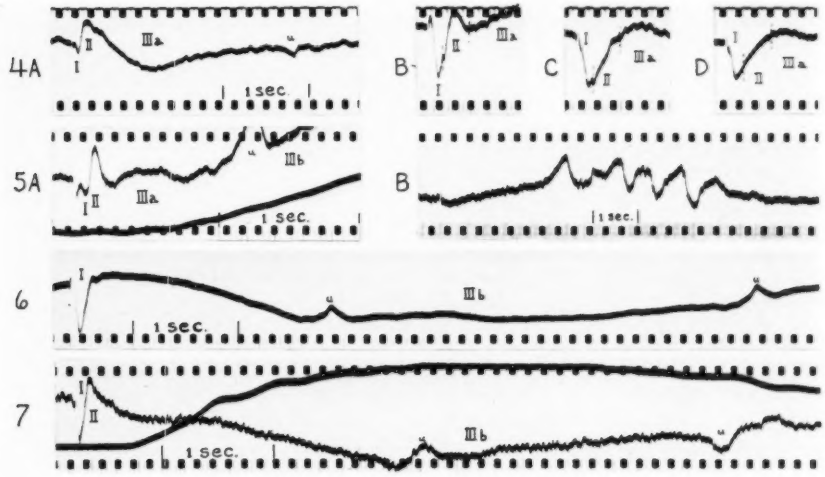
The recording leads were in a few cases Ag-AgCl needle electrodes but, in the majority of cases, the agar-wick non-polarizable electrodes already referred to (cf. Method) were used. One of them, the grid lead, was placed on various parts of the fundus of the bladder or trigonum, and the other, the ground lead, was usually placed on the connective tissue of the leg opposite to the side stimulated, in order to avoid artefacts from spread of stimulus. Maximal shocks were used.

The ureters and the urethra were not tied, so that the urine was free to enter the bladder and to leave during contraction of the fundus. Reflex emptying was, obviously, impossible in the denervated preparation.

The bladder was partially fixed by tying the tip of the fundus to a supporting rod, and was in all cases lifted out of the body cavity.

Myograph records were obtained simultaneously in some cases by attaching a hook to a visibly contracting part of the muscle and placing the recording electrode close to the hook. The time relations of such a record are unreliable, because of the complexity of structure of the bladder.

The electrogram of the bladder is more irregular and complex than that of the nictitating membrane or that of the pilomotors of the tail. One or more of the three potentials described in the previous section may be missing or confused. The component III is so large in some preparations, even with reduced amplification, that I and II appear relatively insignificant. This was true in many of the preliminary experiments, so that II was overlooked. A comparative study of many records shows, however, that II is present in the bladder and that the sequence of potentials is



Figs. 4, 5, 6 and 7. Electric responses from the fundus of the bladder to single shocks applied to the pelvic nerve.

Fig. 4. A, B, C and D. Responses obtained with Ag-AgCl needle electrodes. June 6, 1934.

A. Response obtained early in the experiment, showing I, II and IIIa. *u*, in this and the following records, indicates the potential derived from the ureter (cf. text). μv./mm. = 6.2.

B, C and D. Responses obtained from one position at slightly different times later in the same experiment showing changes in the phases of II. Dotted lines indicate approximately the beginnings of II and IIIa according to their respective latencies. B. μv./mm. = 4.4, C = 4.4, D = 4.8.

Fig. 5. A. Responses obtained with wick electrodes showing II, particularly. I is less clear; IIIa and IIIb are fairly well marked. μv./mm. = 3.6. November 22, 1934.

B. A rhythmic response, IIIa, obtained from the fundus in response to a single shock. Film, much slower. μv./mm. = 16.6 June 12, 1934.

Fig. 6. IIIb is shown without a myogram. I is superimposed on the end of the artefact; II is doubtful. μv./mm. = 3.2. June 28, 1934.

Fig. 7. This record shows IIIb and the myogram occurring at the same time. I and II are fairly well developed. μv./mm. = 11.1. August 8, 1934.

similar to that of the nictitating membrane and of the pilomotors. In several of the records there occurs a potential which accompanies the spontaneous contraction of the ureters and is transmitted to the bladder (see figs. 6 and 7).

Figure 4A represents a response of the bladder to pelvic-nerve stimulation recorded with Ag-AgCl needle electrodes. Of all the bladder records, it is most like the typical electrogram of the nictitating membrane presented in figure 1. The latency of I is about 40σ. This is longer than might be expected, considering the short length of nerve the impulse must travel, but possibly may be accounted for by cooling of the exposed bladder. The spike lasts 70σ and is followed, without pause, by II, which is opposite in sign. The latency of II is 100σ, its duration 160σ. In this record there is a perceptible pause after II, which lasts 80σ and is then followed by IIIa, with a latency of 350σ.

Figures 4B, 4C and 4D show variations in the response obtained from the same preparation later during the course of the experiment. If analyzed with reference to 4A, the various potentials may be recognized as indicated in the figure.

Figure 5A shows a response obtained in another experiment with wick electrodes. I is apparently present, though, as in figure 4A, there is no sharp demarcation between I and II. IIIa and IIIb both occur. The myograph shows contraction beginning at the end of II (cf. fig. 7). Figure 5B illustrates a type of response obtained in several experiments from the fundus of the bladder upon stimulation of the pelvic nerves. It is a series of waves, apparently IIIa, superimposed on a long slow potential, probably IIIb. In this case neither I nor II is present.

Figure 6 shows I with little or no II and a large IIIb. In figure 7 a myogram accompanies the electrical record. In this case, as in figure 5A, the contraction apparently begins at the end of II, with a latency of 480σ. I has here a latency of less than 40σ and a duration of 60σ. II lasts 250σ, and III probably begins 350σ after the stimulus.

The responses of the trigonum upon stimulation of the hypogastric nerves were similar to those obtained from the fundus on pelvic stimulation. I and III have been demonstrated before (cf. fig. 4, Rosenblueth, Leese and Lambert, 1933), while II has been obtained in more recent experiments. They do not therefore require any further illustration.

If all these records are considered, there is evidence for three different potentials which may be obtained in the bladder as a response to a single shock. Due to the complex structure of this organ, the whole series is difficult to obtain in a single record as clearly as in the nictitating membrane. On the whole, however, the electric response of the smooth muscle of the bladder to both pelvic and hypogastric stimulation corresponds closely to the response of smooth muscle of the nictitating membrane and of the pilomotors (cf. sections I and III).

III. Pilomotors of the cat's tail. Wick electrodes were placed on the shaved surface of the tail of the cat or concentric needle electrodes (Adrian and Bronk, 1929) were inserted subcutaneously for recording. The stimulating electrodes were applied to the sympathetic chains in the lumbar region.

The responses obtained with the wick electrodes were wholly comparable to those obtained with the concentric needle electrodes (cf. Rosenblueth, Leese and Lambert, *loc. cit.*, fig. 3B) and to those from the nictitating membrane (fig. 1A). The time relations correspond well to those of the nictitating membrane, though some phases are more prolonged. The

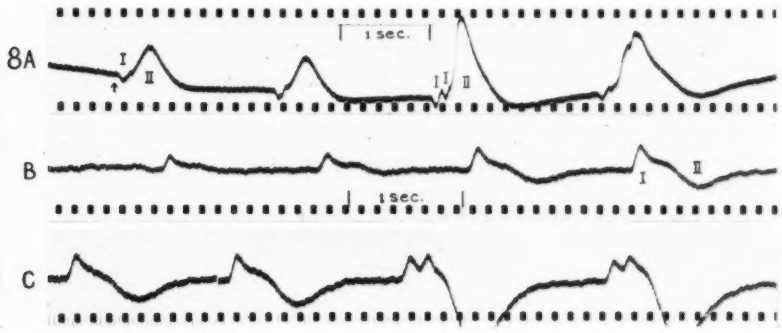


Fig. 8. Electric responses from the pilomotors of the tail to single shocks applied at 1 second intervals to the sympathetic chains. November 2, 1934.

A. A series early in the experiment. Two responses to single shocks and two to 1 and 2 pairs of shocks. μv./mm. = 16.6.

B and C. A continuous series of responses obtained later, showing facilitation and summation (cf. text). μv./mm. = 10.0.

latency of I, longer because of the greater length of nerve between stimulus and muscle, is 115σ, its duration 115σ. The duration of the pause between I and II varied from 40σ in the fresh preparation (fig. 8A) to 140σ later in the same experiment (figs. 8B and 8C). The latency of II is also variable, 230σ or more, its duration 520σ to 600σ. III was not obtained in response to a single shock. In one or two cases, however, a series of shocks produced a long, slow, third wave which resembled IIIb.

Figure 8 (B and C) is a continuous record showing an apparent facilitation in the electric response, i.e., an increased response to a stimulus of the same strength. It is notable that both I and II increase in size in this case. The same figure (A and C) shows the effect of two closely spaced stimuli, separate I waves of the same magnitude and large summated II potentials.

The latencies and durations of the three components of the electrogram in the three muscles studied are summarized in table 1.

The latencies for contraction were as follows: nictitating membrane, 220 to 360σ; bladder (pelvic), 480σ. Brown (1934) has reported a latency of 150σ for the contraction of the nictitating membrane. The latter figure is undoubtedly more accurate than those obtained here with a less sensitive myograph.

Controls. In one of our early experiments upon the bladder, it was observed that a deflection similar to IIIb, the large third potential particularly evident in the bladder (cf. figs. 6 and 7), could be produced by passive movement of the silver-needle grid electrode. This suggested that IIIb might be due to a change of resistance in the recording circuit as the contact of the lead with the naturally polarized tissue was mechanically disturbed during contraction. That the tissue itself was a possible source of a constant potential was indicated by an initial polarization of 4 to 20 mv. in most of the nictitating membrane and bladder preparations. To eliminate the changing polarization of the leads as a possible source of potential and to reduce the amount of contact change of the electrodes, Ag-AgCl,

TABLE 1

	I		II		III	
	Latency	Duration	Latency	Duration	Latency	Duration
Nictitating membrane	40	80–100	120–200	280–440	400–600	1000+
Bladder	30– 50	40– 90	100–120	120–200	320–390	1000+
Pilomotors	60–120	115–140	200–250	520–600		

agar-saline wicks were used in place of the Ag-AgCl needles. With the wicks the only detectable change in contact during contraction was a sliding of the fibers under the agar-saline junction of wick and muscle. Nevertheless, IIIb still appeared as large as before.

In several experiments a thread was attached to the wicks, and the sliding contact was imitated by pulling the wick along the surface, or the area of contact was changed by raising or lowering the wick. Neither type of contact change produced a potential of the same magnitude as that developed during contraction. It seemed apparent, therefore, that the change of resistance dependent on surface contact could be only partially responsible for IIIb, if at all.

As a further test, the tissue was polarized in alternate directions from an outside source through the electrodes (cf. Method). If IIIb depended exclusively upon a change of resistance—whether due to variations in contact of the electrodes or in the shape or blood supply of the tissues during contraction—its sign should reverse with the direction of the polarization (tissue plus outside source). If, on the other hand, IIIb should represent a change in the initial polarization of the tissue, even

though this change would necessarily involve a variation in resistance, its magnitude and sign should be partially independent of the polarization imposed from the outside source.

This test was applied to the bladder in 6 animals and to the nictitating membrane in 2 cases. In 3 of the 8 experiments no reversal took place, although the polarization was carried to 28, 92, and 120 mv., respectively, in the direction opposite to that of the original polarization of the tissue. In 2 other cases a change in the sign of IIIb occurred, but only when the counter-polarization had been carried to 64 and 100 mv., respectively. In 3, finally, reversals occurred close to 0 polarization.

In the majority of the tests the relatively small electrical effects correlated with changes of contact of the grid electrode (passive movement) were found to reverse quite independently of the changes in the response to stimulation. In those cases in which the contact effect was the same in direction as the response to stimulation and reversed with it, the former was always much smaller.

Various other controls such as stretching the bladder, changing its initial distension and the initial tension of the nictitating membrane indicated that the responses were independent of these factors.

The potentials I and II, when present, were never reversed in the preceding experiments.

These controls indicate that I and II do not depend on resistance changes either at the contact of the electrodes on the surface of the muscle or within the muscle tissue, since they were never reversed with a reversal of the polarizing current. II was sometimes displaced from the baseline in one direction or the other (during strong polarization) without a change in sign, when IIIb (which, in this case, began in the middle of II) was reversed. IIIa was also found to be independent of the reversal of the polarizing current. IIIb was almost entirely independent of changes of resistance at the contact of the electrode and muscle. Furthermore, it frequently did not change in sign even when relatively large polarizing currents of opposite direction were applied. We therefore conclude that IIIb, like I, II and IIIa, is due probably to a change in the polarization of the muscle (cf. Bacq and Monnier, 1935).

Discussion. In our previous classification of the potentials obtained from smooth muscle (Rosenblueth, Leese and Lambert, 1933), the spike potential was called the "initial" potential and the long potential associated with contraction, the "delayed" potential. A comparison of the potentials described previously with our present schema indicates that the initial potential was sometimes equivalent to I of our present schema, sometimes a composite of I and II, and that the delayed potential was either II or III. Thus in figure 2 (Rosenblueth, Leese and Lambert, *loc. cit.*) some of the initial potentials are polyphasic and probably com-

posites of I and II, and the delayed potential is III. In figure 4 (*loc. cit.*) the initial and delayed potentials from the bladder are I and III, respectively, whereas, in figure 3 (*loc. cit.*), the delayed potential from the pilomotors of the tail is II.

With the present systematization, there is probably not an absolute equivalence of all phases of the electrograms from the three types of smooth muscle described. In the three muscles, I is of practically the same duration; its latencies correspond well if the differences in the length of nerve for the various preparations are considered. Potential II, on the other hand, is variable in duration, being shortest for the bladder and longest for the pilomotors. The latencies of II with respect to I also vary; it generally follows I, immediately, in the bladder, with no clear separation between the two, whereas, in the nictitating membrane and the pilomotors, there is usually a distinct pause between I and II. The potentials IIIa and IIIb are evident in both the nictitating membrane and the bladder, but have not been clearly identified in the pilomotors.

It is possible that the variability of II may be due to the different conditions of electrical recording in the three preparations because of their structure, rather than to intrinsic differences in the physiological patterns. This may be especially true in the bladder, of which the innervation as well as the structure is complicated (cf. Gruber, 1933).

From the results described here and from the electrograms of cats' uteri reported previously (Rosenblueth, Leese and Lambert, 1933), it appears that no simple schema will cover all the electric responses found in smooth muscle. Thus, the only electric response of the pregnant uterus which we have been able to record (Rosenblueth, Leese and Lambert, *loc. cit.*) is a rhythmic pattern occurring during contraction, whether this contraction appears "spontaneously" or is evoked by repetitive stimulation of the nerves. In terms of the present systematization, these potentials might be IIIa, but a complete analogy cannot be affirmed until further data are available.

There may be some correlation of the type of electrogram with the physiological properties of the different muscles. The nictitating membrane, the pilomotors and the bladder (both pelvic-fundus and hypogastric-trigonum) are neuromuscular systems which respond to single shocks (non-iterative), while the uterus probably requires iterative stimulation. This physiological difference might be responsible for the lack of correspondence in the electrograms.

Monnier and Bacq (1935) studied the initial potentials of the nictitating membrane. They used a KCl electrode to depolarize a region of the muscle and a d'Arsonval-Lapicque wick as an active lead. They do not distinguish the two components I and II described above (figs. 1 to 3) but discuss only an inconstant monophasic response, which decreases in ampli-

tude and disappears after a few stimuli, even when applied at intervals of 1 per second. They conclude that they are dealing with the "action potential" of the membrane—i.e., with the electrical phenomenon corresponding to the spike potential of striped muscle. They summarize in a table the latencies and durations of this potential in four animals (see column headed "Before injection of 933F" in their paper) and give the following figures:

Latencies: 175, 137, 65 and 88σ.

Durations: 41, 333, 57 and 104σ.

A comparison of our latencies and durations of I and II in the nictitating membrane (cf. table 1, p. 154) with these figures is difficult because of the considerable variability in their four observations. Their latencies are, however, all greater than our average for I, and the first two are of the order of magnitude of II, not I. Their records are not clear enough to decide which of the potentials they recorded, I or II, for the stimulus artefacts last well into the responses—a fact which is not surprising, since the stimuli used were discharges from a condenser of a capacity of 11 μF. It is probable, however, that what Monnier and Bacq recorded was not the spike potential I, but II, and that I was entirely masked by the stimulus artefact. If this interpretation of their figures and records is correct, it follows that the several conclusions they derive from the behavior of the "action potentials" of smooth muscle under the influence of diverse drugs are premature.

We disagree with the statement on the inconstancy and fatigability of the initial potentials. In all the cats studied thus far (30) in the present and the previous report (Rosenblueth, Leese and Lambert, 1933) we have always been able to demonstrate I and almost invariably II. Furthermore, we have never encountered with slow frequencies of stimulation the decrease and rapid disappearance of either I or II which Monnier and Bacq stress. A slight equilibration of both I and II is sometimes observed (fig. 1) but a steady state is rapidly attained with no significant subsequent decline. It is possible that the KCl electrode employed in their experiments not only depolarized the underlying region of the muscle, but injured the whole tissue, thus impairing the entire electric response.

The component IIIb is similar in character and time relations to the potentials described by Bacq and Monnier (1935) in the nictitating membrane, the bladder, the uterus and the vas deferens. They conclude that these potentials denote a depolarization of the muscle which precedes and is the cause of contraction. Our experiments support the conclusion that the source of these potentials is a depolarization associated with the contractile process (section IV, p. 155; cf. also Dubuisson, 1934). We do not agree, however, with their conclusion that IIIb precedes the contraction evoked by nerve stimulation. Their records (figs. 1 and 7 in their paper) do not show clearly that the electrogram precedes the mechanogram, nor is

their time scale (seconds) adequate for an accurate analysis which would require discrimination of milliseconds (cf. table 1, Section III). Furthermore, their myograms are isotonic, which are inadequate to reveal the exact onset of contraction. Our own records do not allow any precise conclusion in this respect, for the mechanogram was not sufficiently accurate (cf. Brown, 1934; cf. also p. 154). If IIIb is analogous to the wave "T" of skeletal muscle (Bishop and Gilson, 1927; discussed by Dubuisson, 1934), as we are inclined to believe, it should be strictly simultaneous with contraction. An exact determination of the beginning of IIIb is probably impossible, however, for contraction begins at least as early as the middle of II (cf. figs. 2 and 3), and Brown's latency indicates that in the nictitating membrane contraction starts nearer the beginning of, if not completely coincident with, II (cf. table 1 and p. 150). In any case, therefore, whether IIIb begins simultaneously with contraction or precedes it, its gradual onset would be masked by II. In most of our records, IIIb was not clearly detectable during II and hence is classified as following II (cf. p. 150). Our most direct evidence that IIIb may begin during II is that, in the case of the bladder already described (p. 155), the onset of IIIb was so intensified by the outside source of polarization that the end of II was displaced.

The interpretation of the different components of the smooth-muscle electrograms described above can be attempted only very tentatively, for such a discussion is necessarily speculative in view of the paucity of data available.

The recent knowledge of chemical mediation of autonomic nerve impulses (cf. Cannon, 1933) suggests the following sequence of events when the nerve impulse reaches the effector: *a*, the mediator is liberated in the innervated cells, *b*, it diffuses throughout the muscle (cf. Rosenblueth and Rioch, 1933); *c*, it combines with the receptive substance (Cannon and Rosenblueth, 1933); *d*, contraction ensues, which implies additional chemical changes.

It appears plausible then to correlate I with *a*, as was previously suggested (Rosenblueth, Leese and Lambert, 1933); II possibly with *c* or *d;* and IIIb with *d*. The source of IIIa, the rhythmic response, is unknown at present. It might be possible that the diffusion (*b*) of the mediator from cell to cell is so slow that each wave of IIIa might represent a new set of elements brought into play, but such a suggestion must await further evidence. It is interesting to recall that rhythmic potentials were found also in another autonomic effector, the submaxillary gland (Rosenblueth, Forbes and Lambert, 1933).

SUMMARY

The electric responses obtained from the nictitating membrane upon stimulation of the cervical sympathetic nerve, from the fundus of the

urinary bladder upon stimulation of the pelvic nerve (parasympathetic) and from the pilomotors of the tail upon stimulation of the abdominal sympathetic chains were recorded.

A direct-coupled amplifier allowed a more accurate study of the latencies and durations of the various potentials obtained than had been previously reported (Rosenblueth, Leese and Lambert, 1933).

A systematization of the potentials obtained in response to a single shock into four components, I, II, IIIa and IIIb, is given in place of the previous classification, "initial" and "delayed" potentials, already described.

The potential I corresponds to the former initial potential and the spike potential of striated muscle (cf. figs. 1, 4A and 8A; cf. also Discussion, p. 155).

Potential II was not clearly distinguished from the initial or delayed responses in our previous paper (Rosenblueth, Leese and Lambert, *loc. cit.*). It follows I almost immediately and either slightly precedes contraction or possibly is simultaneous with it (cf. Discussion, p. 158).

IIIa follows II during contraction and may be monophasic, diphasic or polyphasic, becoming repetitive in some cases (cf. figs. 1, 2 and 5B).

IIIb corresponds to the delayed potential previously described (*loc. cit.*). It is a long, slow wave which follows closely the course of contraction (cf. figs. 3 and 7).

Controls indicated that IIIb is not an artefact dependent upon changes of resistance due to changes of contact of the electrodes, or due to changes of shape and blood supply of the tissue during contraction (cf. Controls, p. 154).

The results recently reported by Monnier and Bacq (1935) and Bacq and Monnier (1935) are discussed with reference to the schema presented here (cf. Discussion, p. 156).

A correlation of the potentials I, II, IIIa and IIIb with the physiology of chemical mediation is tentatively suggested (cf. Discussion, p. 158).

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METHYLENE BLUE AND HEMOGLOBIN DERIVATIVES IN ASPHYXIAL POISONING[1]

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The suggestion that methylene blue should be used as an antidote in cyanide and carbon monoxide poisoning was first made by the writer (Brooks, 1932, 1933), and it was first successfully used in both types of poisoning by Geiger (1932, 1933). Since that time there have appeared in the literature many articles as to the mode of action of this dye in both types of poisoning.

One theory advanced by Hug (1933), and favored by Wendel (1933, 1934), Henderson (1933), Haggard and Greenberg (1933) and Hanzlik and Richardson (1934) is based upon the assumption that methylene blue, and also nitrites, produce methemoglobin in the bloodstream, and this subsequently unites with CN to form cyanmethemoglobin. This stable compound is then spoken of as having withdrawn the cyanide from active participation in body processes, thus accounting for the favorable influence of both agents in cyanide poisoning. But because of this same line of reasoning Henderson (1933) and Haggard and Greenberg (1933) postulate that methylene blue should not be used in cases of carbon monoxide poisoning, since by presumably changing oxyhemoglobin to methemoglobin it would act as a synergist rather than as an aid to resuscitation.

However, this hypothesis does not explain certain experimental observations made by the writer, and further studies described in the present paper show why it is not properly applicable to the therapeutic use of methylene blue. The question revolves mainly around the cyanide derivatives of hemoglobin. When washed red blood cells from fresh defibrinated blood were used *in vitro* and in experiments *in vivo*, it was always found by the writer that the blood became bright red on the addition of cyanide. Kobert (1900) and also Cook (1928) noted this with defibrinated blood. On the other hand, Barnard (1933) found that when cyanide was added to crystallized methemoglobin, a dark brown substance was formed. He found further that the absorption spectrum of this substance was identical with that for methemoglobin, the primary maximum of both lying at 540 mμ.

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The confusion which seems to prevail in the literature concerning the identity of the substance formed when CN is present in blood appears to have resulted from failure to recognize the existence of these two distinct derivatives of hemoglobin, and of the acid and alkaline forms of methemoglobin. The pH of a methemoglobin solution, as pointed out by Haurowitz (1924), plays an important rôle in the spectrophotometric picture. Both acid and alkaline solutions of methemoglobin have a primary maximum at 543 mμ and a secondary maximum at 576 mμ, the latter resulting from the presence of small amounts of oxyhemoglobin as an impurity. But acid methemoglobin has an additional absorption band at 630 mμ which is absent in alkaline solutions. Many investigators who deal with the absorption bands of methemoglobin fail to distinguish between these two or their intermediate forms.

The failure to recognize the two possible cyanide derivatives of hemoglobin has arisen largely from neglect of two important factors which determine which of the two substances shall be formed in any given experiment. These factors are:

1. *The presence in blood of substances other than pure oxyhemoglobin.* One set of investigators has used defibrinated blood and another, crystals of oxyhemoglobin or methemoglobin. While it is the object of scientific experimentation to isolate separate reactions in their simplest form, nevertheless, it is not justifiable in seeking to explain a mechanism affected by many factors to neglect all but one of these factors.

Warburg, Kubowitz and Christian (1931) recognizing this, tried experiments both with defibrinated blood and with hemoglobin crystals. They attributed the differences between these systems to the presence or absence of glucose and other reducing substances. Wright, Conant and Kamerling (1933) have shown that unsaturated fatty acids are probably the principal reducing substances present in blood. The writer has also shown (1934) that the absorption spectra obtained on addition of various agents to suspensions of red blood cells in the presence of glucose *in vitro* are different from those in the absence of glucose. Glucose inhibits the formation of methemoglobin, and the presence of glucose and other reducing substances in normal blood inhibits the methemoglobin formation which occurs when pure crystallized hemoglobin derivatives are used.

The presence of these reducing substances in the blood has been ignored by those writers who quote Warburg in confirmation of their idea that methylene blue forms methemoglobin *in vivo*. Methylene blue can transform hemoglobin into methemoglobin only if these reducing substances are absent. In the blood stream they are present, and therefore one cannot explain *in vivo* experiments by isolating pure hemoglobin and drawing conclusions from experiments on that. Since oxyhemoglobin exposed to air gradually changes to methemoglobin unless glucose or other reducing

substances are present, any protracted analytical method for determining the relative amounts of oxyhemoglobin and methemoglobin is accurate only when allowance is made for the oxidation which occurs during the analysis.

2. *The concentrations and proportions in which the different reagents are used.* The writer's conclusions have been based upon experiments in which approximately equimolal amounts of reagents were used, while the amounts used by some of the other investigators are either not reported or are not comparable. Haurowitz (1931) for example, gives the concentration of KCN used but not that of methemoglobin. Barnard (1933) on the other hand was not working on toxicity and purposely chose conditions totally different from those obtaining *in vivo*. He used 1000 to 2000 moles of KCN to each mole of hemoglobin. It will be shown later that such a concentrated solution of KCN changes the absorption spectrum of hemoglobin. His solutions also did not contain glucose or other reducing substances, and his experiments are therefore not comparable to *in vivo* experiments, and cannot be quoted in support of the doctrine that in the body the addition of methylene blue and cyanide to hemoglobin produces cyanmethemoglobin.

In the present paper, the results obtained under different conditions of purity, relative concentration, etc., have been compared with the above considerations in mind.

Experiments. In all the experiments modifications of the hemoglobin system of blood pigments were produced by the introduction of one or more of the agents mentioned below. In all cases samples were taken for spectrophotometric study of the absorption spectra on the resulting derivatives of hemoglobin.

Samples of blood were obtained at the times and in the manner described below. One cubic centimeter of whole or defibrinated blood was diluted to 100 cc. with 0.4 per cent NH_4OH to form an optically clear alkaline solution. In experiments employing crystalline oxyhemoglobin the final solution contained an approximately equal concentration of hemoglobin derivatives also dissolved in 0.4 per cent NH_4OH. Except in the case of reduced hemoglobin, the samples were thoroughly aerated.

Determinations of the extinction coefficients at different wave lengths were made, several readings being taken, using both right and left hand columns, and calculating the mean of these. A layer of solution 1 cm. deep was used. The extinction coefficients may be read directly from the graduated circle of the 1928 Bausch and Lomb spectrophotometer used. The usual further precautions (described in the hand book accompanying the instrument) were observed.

The extinction coefficients between 500 and 640 mμ were determined at intervals of 10 mμ (or less in the region of the absorption maxima) for one

or more typical experiments of every kind, thus establishing the type of absorption spectrum obtained. In succeeding qualitatively similar experiments only the extinction coefficients at 540 and 560 mμ were observed, these being all that were needed to determine the proportions of reduced and oxyhemoglobin, or methemoglobin and oxyhemoglobin. The ratio, R, of these two extinction coefficients in such mixtures has been determined by Ray, Blair, and Thomas (1932) and is given in table 1 below, together with the corresponding proportions of the hemoglobin derivatives. This ratio, R, was the same in samples diluted with pure water as in those diluted with 0.4 per cent NH_4OH.

Readings on the spectrophotometer were made immediately on obtaining the blood and were completed within ten minutes, or considerably less if only the extinction coefficients at 540 and 560 mμ were to be determined. The extinction coefficients so determined are accurate to within less than 2 per cent. In many cases identical readings were obtained in several successive trials, even though the necessary settings must be made "blind" and the results read off afterwards.

Since the details of dosage and manipulation differed in different sections of this work they are described in connection with the appropriate section. The experiments may be divided conveniently into two groups: those in which pure crystallized hemoglobin preparations were used (section 1) and those in which defibrinated blood *in vitro* (section 2a) or whole blood *in vivo* (section 2b) were used.

SECTION 1. CRYSTALLIZED OXYHEMOGLOBIN. *Methods.* Undenatured crystallized oxyhemoglobin was obtained by defibrinating fresh horse blood, washing several times with 0.9 per cent NaCl solution, decanting, bringing to original volume with distilled water, adding 20 per cent ice-cold ethyl alcohol and keeping at freezing temperature. Abundant crystals are produced which were kept moist to prevent denaturation.

There is a slight error in weighing these crystals owing to the contained moisture, but this is not significant in this connection. A 0.0001 M solution of oxyhemoglobin was made up from these crystals and to it was added 1 to 10 times as many moles of KCN. The molecular weight of hemoglobin here and throughout this paper is assumed to be 66,000 (Bickery and Leavenworth, 1928). The resulting solution was allowed to stand at 23°C. In other experiments KCN was added in large excess, viz., about 1000 moles per mole of oxyhemoglobin, so as to determine whether such conditions were comparable with those when smaller cyanide doses are used, as in the usual cases of cyanide poisoning.

Results. When from 1 to 10 moles of KCN per mole of crystallized oxyhemoglobin was added to a solution of the latter, the absorption spectrum remained unchanged for several hours at room temperature. After this a brown color gradually became perceptible and methemoglobin

appeared as was shown by the spectrophotometer. When however, about 1000 moles of KCN was used per mole of oxyhemoglobin, the solution immediately turned brown and had the absorption spectrum of methemoglobin. Barnard (1933) justifiably concludes that this condition is due not to the presence of methemoglobin alone, but to the formation of a cyanide derivative which he calls cyanmethemoglobin, and which has the same absorption spectrum as methemoglobin. This amount of KCN is within the range used by Barnard who, starting with crystallized methemoglobin, found the methemoglobin absorption spectrum to persist upon the addition of KCN. Haurowitz (1931) also used crystallized methemoglobin and upon adding KCN obtained a solution whose absorption maximum lay at about 540 mμ, which agrees with that given by Barnard. His ratio of KCN to methemoglobin was probably much the same as Barnard's, although as previously stated, his failure to give the concentration of his methemoglobin solution makes it impossible to be sure of this.

It will be shown later (section 2a) that if similar large amounts of KCN are added to fresh defibrinated blood, the suspension soon turns brown. The change in that case is slower than with crystallized oxyhemoglobin, presumably because of the delaying influence of reducing substances.

Section 2a. Defibrinated blood in vitro. *Methods.* Fresh defibrinated sheep blood was centrifuged, washed several times with 0.9 per cent NaCl and brought to the original volume with NaCl. To 2 cc. of blood was added 0.2 cc. of 0.33 M $NaNO_2$, or an equivalent amount of KCN, or both, or 0.1 cc. of 0.01 M methylene blue. Rat and rabbit bloods gave essentially similar results. Oxyhemoglobin was formed by shaking the blood vigorously with air or oxygen; reduced hemoglobin by the addition of Stokes' reagent or $Na_2S_2O_4$ according to the usual procedures; methemoglobin, by the addition of $NaNO_2$. Enough $NaNO_2$ was used to change 88 per cent of the oxyhemoglobin into methemoglobin. The chemically equivalent amount of KCN was used, but not of methylene blue because it is a reversible oxidation-reduction dye and is able, by repeated cyclic reduction and oxidation, to oxidize many times the equivalent amount of another suitable substance, such as hemoglobin (Warburg, Kubowitz and Christian, 1930). In calculating how much of the various agents to use it was assumed that 100 cc. of blood contained 13.9 grams of hemoglobin (Macleod, 1928). This latter figure varies with the species, the condition of the animal, etc., but the variation is not significant for the purpose of these experiments.

Results. Table 1 gives the results of this set of experiments. In the case of oxyhemoglobin alone, the appropriate value of R was obtained. An identical absorption curve and R value were obtained for oxyhemoglobin plus KCN. The preparation which, because of the presence of $NaNO_2$, showed 88 per cent methemoglobin before the addition of KCN, gave an

absorption curve and R value characteristic of that of pure oxyhemoglobin immediately after the addition. Similar results were obtained when KCN was added to reduced hemoglobin. These readings were the same two hours later, but 48 hours later the absorption spectra of all solutions except no. 2 (table 1) showed the presence of some methemoglobin. Very large additions of KCN to defibrinated blood hastened the appearance of methemoglobin, so that when the ratio of KCN to methemoglobin was approximately 1000 moles to 1, methemoglobin could be detected after a few minutes. However this production of methemoglobin *in vitro* has no significance in the therapy of cyanide poisoning, both because of the overwhelming amount of KCN required, and because of the presence *in vivo* of greater reserves of reducing substances.

The experiments illustrated in table 1 were done at room temperature, but even when the blood cell suspensions containing KCN were warmed to

TABLE 1

The effect of KCN on different derivatives of hemoglobin in defibrinated blood in vitro

The resulting proportion of hemoglobin is calculated from the ratio, R, of the extinction coefficients at 540 and 560 mμ.

NUMBER	FORM OF HEMOGLOBIN	ADDITION	R	PER CENT OXYHEMOGLOBIN
1	Oxyhemoglobin	None	1.63	100
2	Oxyhemoglobin	KCN	1 62	
3	Methemoglobin	None	1 28	12
4	Methemoglobin	KCN	1 63	
5	Reduced hemoglobin	None	0 849	0
6	Reduced hemoglobin	KCN	1 62	

40°C. and allowed to stand for some time, the oxyhemoglobin spectrum persisted.

Figure 1 shows absorption curves in more detail. Curve *A* is that for oxyhemoglobin alone; curve *B*, when $NaNO_2$ and KCN have been added to oxyhemoglobin; curve *C*, when KCN alone has been added to oxyhemoglobin. The curves are essentially identical, having maxima at the same positions and the same R values. In figure 2, curve *A* is that for 75 per cent methemoglobin produced by the addition of $NaNO_2$, and curve *B*, that for a similar solution to which KCN has been added. Curve *A* shows a primary maximum at 543 mμ, and a secondary maximum at 576 mμ indicating the presence of some oxyhemoglobin. In figure 3, curve *A* is that for completely reduced hemoglobin produced by the action of Stokes' reagent; it has a maximum at 555 mμ; curve B, for the same solution after KCN had been added, is again identical with that for oxyhemoglobin.

The experiments shown in figures 1 to 3 and table 1 then show that when

cyanide is added to defibrinated blood, no matter which of the three forms the hemoglobin is in,—reduced, oxy- or met-, there forms immediately a hemoglobin derivative which has the typical absorption spectrum of oxyhemoglobin. From other experiments we know that it is not oxyhemoglobin and it is therefore impossible to deduce from the R values in table 1 how much oxyhemoglobin, if any, is present. It is for this reason that no figure is given for per cent oxyhemoglobin opposite nos. 2, 4 and 6 in the table.

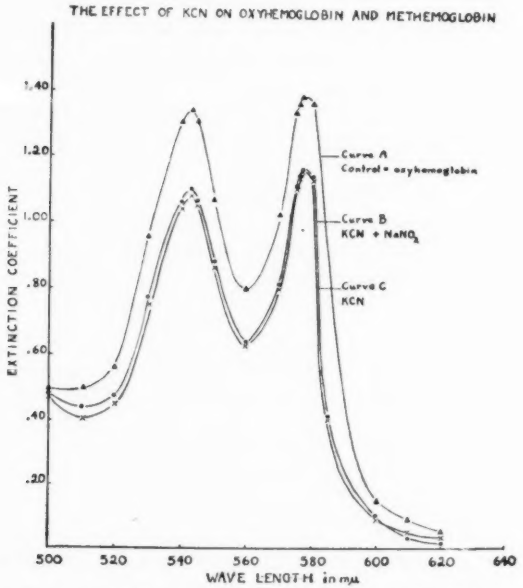

Fig. 1. The effect of KCN on oxyhemoglobin or methemoglobin in defibrinated blood *in vitro*. Abscissae are wave lengths in mμ and ordinates are extinction coefficients. Curve *A*, control, oxyhemoglobin alone; curve *B*, KCN added to methemoglobin formed by $NaNO_2$; curve *C*, KCN added to oxyhemoglobin. Concentrations given in text.

Figure 4 illustrates the formation of oxyhemoglobin when glucose is added to a red blood cell suspension containing methemoglobin. $NaNO_2$ was added to defibrinated blood producing 55 per cent methemoglobin (curve *A*). The cells, which were brown in color, were then washed with a 0.9 per cent NaCl solution, centrifuged, the supernatant fluid decanted and 1 per cent glucose added. The spectrophotometer showed that a large part of the methemoglobin had been reduced to oxyhemoglobin so that now only 17 per cent methemoglobin was present (curve *B*). The same

thing can be shown if glucose is added first and then $NaNO_2$. The fact that the methemoglobin is not all reduced to oxyhemoglobin has been commented on by Warburg, Kubowitz and Christian (1930, 1931) and by Brooks (1934).

SECTION 2B. WHOLE BLOOD IN VIVO. *Methods.* Experiments were done with over 200 rats, 61 rabbits and one dog. Dosages were determined upon the basis of generally accepted estimates of blood volume and hemoglobin content of the blood, e.g., for the rabbit, the blood was assumed to make up 5.82 per cent of the body weight (Gulbertson, 1934), and to contain 13.9

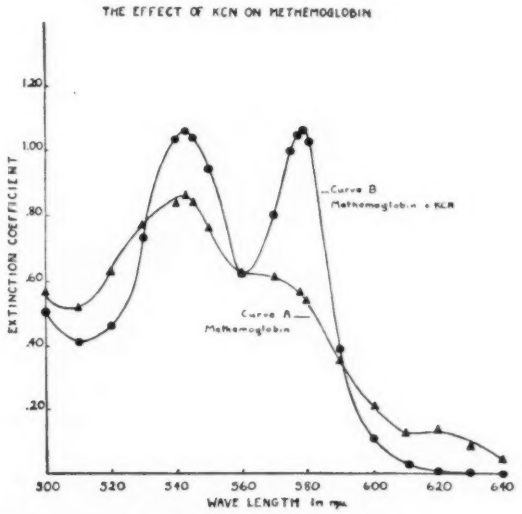

Fig. 2. The effect of KCN on methemoglobin in defibrinated blood *in vitro*. Abscissae are wave lengths in mμ and ordinates are extinction coefficients. Curve *A*, methemoglobin formed by $NaNO_2$; curve *B*, the same to which KCN had been added, showing formation of oxyhemoglobin-like curve. Concentrations given in text.

grams of hemoglobin per 100 cc. (Macleod, 1928). It was found however that the amount of $NaNO_2$ required to produce a given proportion of methemoglobin was greater than has usually been reported, and also greater than might be predicted on the basis of chemical equivalence. Some reasons for this have been discussed by the writer (Brooks, 1934).

The reagents were in all cases dissolved in 0.9 per cent NaCl solution. They were injected into the rats intraperitoneally or into the jugular vein. In the latter case the animals were first anesthetized with ether and the jugular vein exposed. The proportion of hemoglobin transformed to

methemoglobin varied with the amount of $NaNO_2$ injected about as follows: 0.002 gram per 100 grams body weight intraperitoneally gave 15 per cent methemoglobin; 0.03 gram gave 60 to 85 per cent. These proportions, which represent the maxima of methemoglobin formation, were observed at from 2 to 15 minutes after injection, using blood obtained by heart puncture or decapitation.

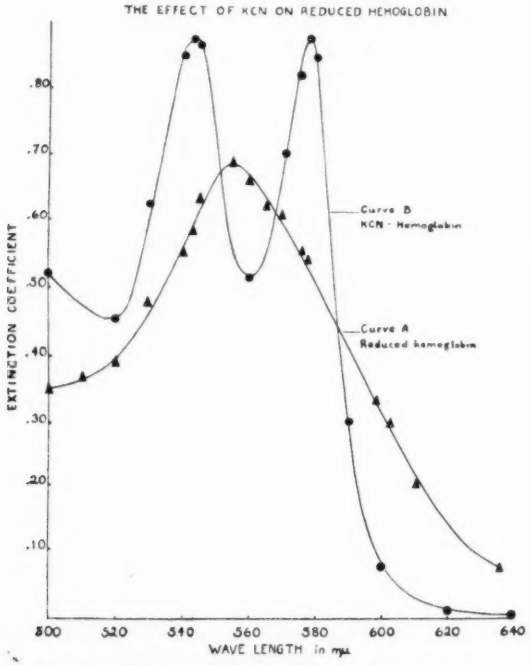

Fig. 3. The effect of KCN on reduced hemoglobin in defibrinated blood *in vitro*. Curve *A*, reduced hemoglobin alone; curve *B*, KCN added to reduced hemoglobin, showing formation of oxyhemoglobin-like curve. Concentrations given in text.

KCN was given in doses of from 0.28 to 2.8 mgm. per 100 grams body weight, or roughly 1 to 10 m.l.d. Excessive doses were used purposely to see whether they would produce different hemoglobin derivatives.

Methylene blue was given as 0.01 or 0.1 per cent solution, 1 cc. per 100 grams body weight.

When rabbits were used, the reagents were injected into an ear vein, and venous blood samples taken from the opposite ear after intervals varying from a few minutes up to 2 hours. The dosages used per 100 grams body weight are: $NaNO_2$, 0.002 to 0.015 gram; KCN, 0.00028 gram; methylene

blue, 0.1 cc. of 0.01 per cent solution; glucose, 0.1 cc. of 1 per cent solution either once or twice with an interval of a few minutes. Control animals received either no injection, or amounts of 0.9 per cent NaCl solution corresponding to the above. The injection of NaCl solution alone did not produce any methemoglobin.

Experiments were also tried with rabbits in whose blood methemoglobin had been produced by the injection of phenylhydroxylamine (0.198 gm. of freshly prepared crystals dissolved in 10 cc. of 0.9 per cent NaCl solution for a rabbit weighing 4.1 kgm.) or by rendering them unconscious by

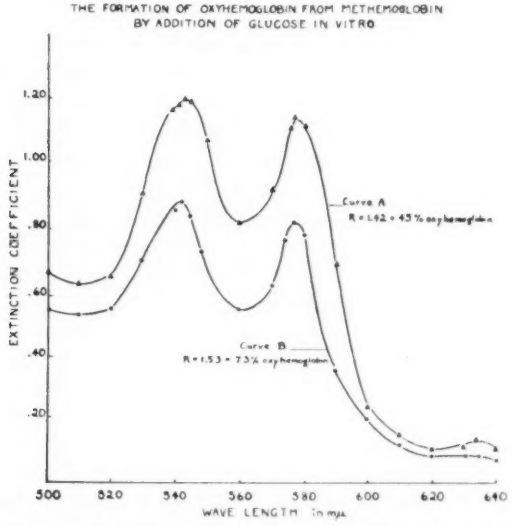

Fig. 4. The effect of the addition of glucose *in vitro* in defibrinated sheep's blood containing 55 per cent methemoglobin, produced by $NaNO_2$. Curve *A*, before addition of glucose; curve *B*, after. In this case methemoglobin was not completely reduced to oxyhemoglobin. Abscissae are wave lengths in mμ and ordinates are extinction coefficients.

inhaled amylnitrite. The results did not differ in any material way from those in which $NaNO_2$ was used, and are therefore not further mentioned.

Rabbits were more satisfactory than rats because blood samples, including a control before injection, could be taken from a single animal repeatedly and the appearance and disappearance of methemoglobin thus followed throughout its whole course. The higher $NaNO_2$ dosage required by rabbits for a similar percentage of methemoglobin production is probably due to the presence of more reducing substances in their blood than in that of rats.

Results. The experiments described in this section were designed to show under what conditions methemoglobin occurs in the blood stream in living animals; they are summarized in table 2. The last column in this table gives the proportions of methemoglobin in the blood as indicated by the R values. In addition to control experiments with and without injection of 0.9 per cent NaCl, experiments were done with $NaNO_2$, glucose, methylene blue and KCN either singly or in various combinations. Both rats and rabbits were employed and since there was no difference in the results with these 2 species, except in dosage of $NaNO_2$, which does not

TABLE 2

The action of various agents upon the production of methemoglobin in vivo

The mean of the results of all the experiments of any given set is used except in the case of sets 3 and 4 in which the extremes of variation are given. The proportion of methemoglobin present is calculated from the ratio, R, of the extinction coefficients at 540 and 560 mμ.

EXPERIMENTAL SET	SUBSTANCE INJECTED	NUMBER OF ANIMALS			R	PER CENT METHEMOGLOBIN
		Rats	Rabbits	Dogs		
1	Control, no injection	20	10		1.62	0
2	Control, 0.9% NaCl	5	2		1.62	0
3	$NaNO_2$, small doses	30	5		1.58–1.55	10–20
4	$NaNO_2$, large doses	30	5		1.33–1.28	75–88
5	Methylene blue	20	4		1.63	0
6	Methylene blue and $NaNO_2$ (all doses)	10	8		1.62	0
7	KCN, all doses	20	6		1.64	0
8	KCN and $NaNO_2$ (all doses)	40	8		1.63	0
9	KCN and methylene blue	10	3		1.63	0
10	$NaNO_2$ (all doses) and glucose	10	5		1.66	0
11	Glucose, then $NaNO_2$	10	5		1.67	0
12	Control, before injection			1	1.61	0
13	Methylene blue alone				1.62	0
Total animals		206	61	1		

concern the ultimate aim of these experiments, the R values for all experiments of a given type were used to calculate the mean values given in the table. The total range of variation of the R values within a given set of experiments varied in different sets between 1 per cent and 5 per cent. In sets 3 and 4 the range of variation is given, since this shows the range of variation in methemoglobin production. It was not always possible to predict just how much methemoglobin would be produced by a given dose of $NaNO_2$. This was, however, always sufficient to demonstrate the effect of the reagents upon it, and aside from this requirement, the exact amount of methemoglobin was immaterial in these experiments.

One experiment was done with a dog to justify broader generalization of the conclusion drawn from the experiments with rats and rabbits as to the effects of methylene blue on hemoglobin *in vivo*.

Table 2 shows the absence of methemoglobin in normal animals or those given 0.9 per cent NaCl solution, and its presence after injection of $NaNO_2$, the amount produced varying with the dosage. In addition it shows that:

1. Methylene blue produces no methemoglobin in normal blood of rats, rabbits or dogs (sets 5, 12 and 13); and that if methemoglobin is already present in blood, methylene blue reduces it to oxyhemoglobin (set 6). It is important to note in this connection that the methylene blue does not in these experiments obscure or alter by its own absorption the significant regions of the oxy- and methemoglobin absorption spectra.

Because of the fact that dogs are generally regarded as reacting more like man than do rats or rabbits, the methylene blue experiment was also tried on a dog. The animal weighed 23.5 kgm., and 16 cc. of a 1 per cent methylene blue solution dissolved in 0.9 per cent NaCl solution was injected into its femoral artery. This is equivalent to the customary clinical dose. Before the injection an R value of 1.61 was found; the R value 15 minutes after injection was 1.63, and 1 hour after 1.62. There was therefore no methemoglobin formation.[2]

2. KCN changes all the hemoglobin into the substance giving the oxyhemoglobin spectrum, regardless of whether oxyhemoglobin (set 7) or methemoglobin (set 8) is originally present. The methemoglobin spectrum, if originally present, disappears completely in the presence of cyanide.

3. KCN also produces the same effect when methylene blue is present (set 9). Since methylene blue does not produce methemoglobin under these circumstances, this was to be expected. Even if methemoglobin had previously been produced by administration of $NaNO_2$ it would have been reduced to oxyhemoglobin by the methylene blue as shown in set 6. This point is demonstrated in more detail by figure 5 which represents a single typical pair of experiments. Curve *A* shows the absorption spectrum of the blood of one rabbit previous to administration of methylene blue. This is a typical spectrum for normal blood. Curve *B* is the absorption spectrum of the same animal after intravenous injection of 1 cc. of a 0.01 per cent solution of the dye per kilogram body weight. This corresponds to the usual clinical dose. No significant change occurred. Curve *C* is the absorption spectrum of blood of another rabbit receiving the same dose of methylene blue just after an intravenous injection of 0.0028 gram of KCN per kilogram body weight. For all these curves the R values lie between 1.61 and 1.62, which shows that no methemoglobin was present. The same spectra characterized blood samples taken at intervals up to 2

[2] I wish to express my thanks to Prof. Eric Ogden and Mr. A. R. Teather of the Department of Physiology of this University, who made the injections.

hours after injection. It is evident that methemoglobin does not appear in the blood of animals given methylene blue after cyanide poisoning sets in.

Differences in the absolute values of the extinction coefficients in similar experiments are due to differences in the concentration of blood in the samples, or in some cases to differences in hemoglobin content of the blood of different animals. Slight differences in the relative values of the extinction coefficients at 576 and 543 mμ are of random occurrence in both

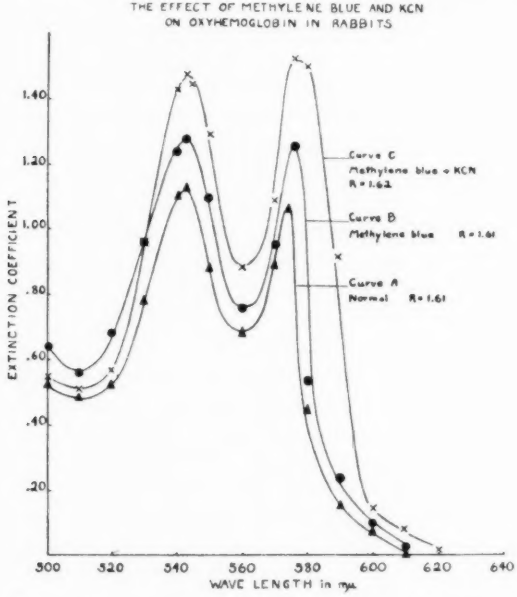

Fig. 5. The effect of intravenous injection of methylene blue and KCN into rabbits. Curve *A*, control, before injection; curve *B*, when methylene blue alone is injected; curve *C*, when methylene blue and KCN have been injected. Abscissae are wave lengths in mμ and ordinates are extinction coefficients. Concentrations given in text.

normal and treated animals and do not appear significant in these studies. Their cause is not known.

Figures for other combinations of reagents will not be given as they are in every respect essentially similar to figure 5.

4. Glucose produces the same effect on the absorption spectrum of methemoglobin containing blood as does KCN. In this case however, the effect is due to the reduction of methemoglobin to oxyhemoglobin (set 10). Figure 6 shows a typical experiment of this type, in which enough $NaNO_2$ was given to produce 15 per cent of methemoglobin (curve

A). Then glucose was injected and subsequent examination showed the complete disappearance of the methemoglobin (curve *B*). Similarly, the injection of glucose prevents the oxidation of oxyhemoglobin to methemoglobin by $NaNO_2$ injected just afterwards (set 11). It was noted that in this set of experiments the veins of the rabbit never turned dark in color on injection of $NaNO_2$, as they did when no glucose had been injected.

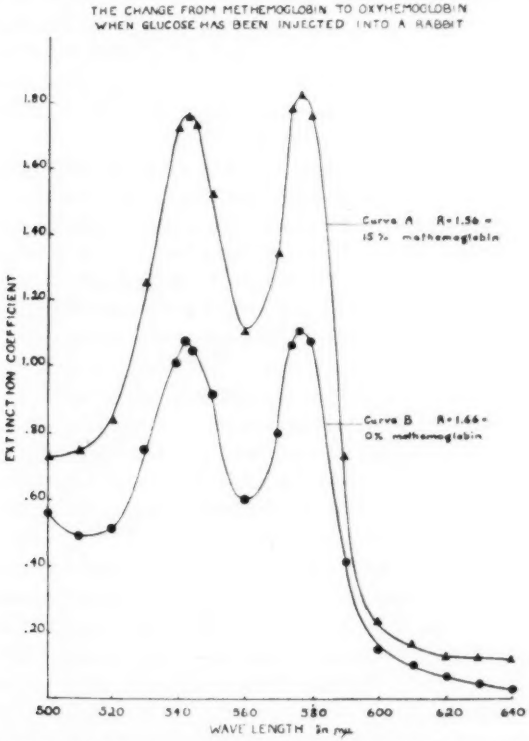

Fig. 6. The effect of an intravenous injection of glucose into a rabbit whose blood contained 15 per cent methemoglobin produced by $NaNO_2$. Curve *A*, before; curve *B*, after injection of glucose. Abscissae are wave lengths in mμ and ordinates are extinction coefficients. Concentrations given in text.

Presumably this effect might be overcome by the use of overwhelming $NaNO_2$ doses, but this would be without significance for the problem under study. These last results have been reported in a preliminary paper (Brooks, 1934).

It is desirable to comment upon the fact that in table 2 there appear some values of R which exceed 1.618, the value assigned by Ray, Blair and

Thomas (1933) for the hemoglobin-oxyhemoglobin system in equilibrium with air at atmospheric pressure. This should theoretically contain almost 100 per cent oxyhemoglobin, and possibly the higher values may represent higher proportions of oxyhemoglobin, or of the cyanide derivative with the same absorption spectrum. However, the high R values were of random occurrence in control animals and animals treated in all different ways, and their significance can not be evaluated at present.

Abnormally low R values were also occasionally found in control animals, seeming to signify the presence of as much as 10 per cent methemoglobin in their blood. Such animals and the corresponding data were discarded. The precise significance of these cases is also unknown, unless they indicate an abnormal condition in the animal.

Discussion. It appears that by the action of CN upon hemoglobin, oxyhemoglobin or methemoglobin, there may arise either of two distinct substances, with absorption spectra like oxyhemoglobin and methemoglobin respectively. The two terms, cyanhemoglobin and cyanmethemoglobin have been applied more or less indiscriminately to these compounds, and great uncertainty has existed as to their nomenclature and relationships. The nature of the union between the CN and the pigment has also been the subject of much speculation. Many workers have tried to deduce from this inadequate basis, hypotheses and dicta as to the toxicology and therapy of CN and other asphyxial agents.

The present paper contributes to a solution of these problems by defining the conditions under which the two substances arise and the reasons therefor; by discussing their probable structural relationship, and by applying this knowledge to the therapeutic problem.

It was found that the bright red oxyhemoglobin-like substance which we shall tentatively call cyanhemoglobin always appeared when KCN in proportions comparable to or even considerably exceeding those usually encountered in fatal poisoning of mammals was given *in vivo* or added to red blood cells *in vitro*. This was true regardless of whether reduced hemoglobin, oxyhemoglobin or methemoglobin predominated initially. The characteristic color and absorption spectrum of cyanhemoglobin resulting from the addition of KCN to oxyhemoglobin in defibrinated blood was unaltered for days at room temperature. However if crystallized oxyhemoglobin and KCN were used in similar amounts, the substance having the color and absorption spectrum of methemoglobin appeared in appreciable concentration within 12 hours. We shall refer to this substance as cyanmethemoglobin. Barnard (1933) has shown, as previously mentioned, that if KCN in large excess be added to crystalline methemoglobin, then cyanmethemoglobin is formed immediately.

The experiments described in the present paper show that methylene blue in the amounts recommended for therapeutic use does not produce

methemoglobin either *in vivo* or in defibrinated blood *in vitro*. Similarly if cyanide is also present, methylene blue does not lead to cyanmethemoglobin formation as has been postulated by various authorities. Instead, cyanhemoglobin with its oxyhemoglobin-like spectrum is produced just as though no methylene blue were present. The assumption that methylene blue owes its therapeutic effect to its capacity to form methemoglobin is therefore without experimental support. Henderson (1933), for example, in a paper entitled "Methylene blue, a synergist" has condemned my suggestion of methylene blue as an antidote for CO poisoning on the basis of this groundless idea. Haggard and Greenberg (1933) make the same mistake.

The reason why different products are formed under the different conditions described above is only partially understood. One factor appears to be the presence of reducing substances *in vivo* and in defibrinated blood.

This explanation may be correlated with the work of Conant (1923) and of Conant and Fieser (1924) who have shown that oxyhemoglobin and methemoglobin form a reversible oxidation-reduction system, in which the ferrous iron of oxyhemoglobin is oxidized to ferric iron in methemoglobin. This system has a well defined potential varying logarithmically with the relative concentrations of oxyhemoglobin and methemoglobin. Substances having a high reducing potential would as a result poise the system at an E_h at which no appreciable amounts of methemoglobin could exist.

Since the spectra of the two cyanide compounds are identical with oxyhemoglobin (which has ferrous iron) and methemoglobin (which has ferric iron) and since the same factors favor or hinder the formation of the spectrally similar forms in the two cases, we conclude that the iron is in the same valence state when the spectra are the same, that is, cyanhemoglobin and oxyhemoglobin have ferrous iron, and cyanmethemoglobin and methemoglobin have ferric iron. The valence of the iron in these compounds determines the valence electron configuration around the iron. This in turn is known to affect the absorption spectrum of the compound. This does not necessarily imply that substances with identical spectra are chemically identical; they may differ in constitution provided only the constraints upon the electrons responsible for absorption of visible light are not thereby altered.

Another detail of the structure of cyanhemoglobin which is of interest is the apparent fact that like oxyhemoglobin it is an oxygenated substance. Thus Cook (1928) has shown that no free oxygen is given up when oxyhemoglobin is transformed to cyanhemoglobin. Although it has not been definitely shown that the oxygen is not given up in some combined form, still it seems very probable that cyanhemoglobin, like oxyhemoglobin, contains 2 atoms of oxygen in its molecule for each atom of iron. Presumably the presence of this oxygen has the same effect upon the forces

constraining the electrons responsible for color in cyanhemoglobin as it does in oxyhemoglobin, thus differentiating both from reduced hemoglobin; furthermore the cyanide appears to have no effect on these forces. This raises two questions, namely: first, the nature of the union between cyanide and oxyhemoglobin and second, the reason for the appearance of the cyanhemoglobin spectrum upon the addition of cyanide to other derivatives of hemoglobin.

Available evidence upon the latter point is inadequate, but the nature of the union between cyanide and the various forms of hemoglobin has been studied by several authors and enough is known to justify tentative hypothesis. Barnard (1933) using crystallized methemoglobin and KCN found that the addition of cyanide to the system lowered its redox potential. Since the removal of hemoglobin raised the potential, and the removal of methemoglobin lowered it, he concludes that cyanide removes the oxidant (methemoglobin) from the system in an inactive, perhaps un-ionized, form, thus lowering the redox potential of the system. This study evidently concerns cyanmethemoglobin and not cyanhemoglobin. It would be of interest to study in the same way the effects of cyanide under conditions such that cyanhemoglobin was the principal product. It must be admitted, however, that the electrometric method offers no direct evidence as to the nature of the cyanide union.

On the basis of chemical evidence Haurowitz (1935) has come to the conclusion that the cyanide ion combines with the ferric iron of methemoglobin, but that no combination is possible between cyanide and the ferrous iron of oxyhemoglobin or reduced hemoglobin. The appearance of a reduced hemoglobin absorption spectrum upon reduction of cyanmethemoglobin by $Na_2S_2O_4$ seemed to Haurowitz to show that reduction of the iron atom drove cyanide out of the molecule leaving only reduced hemoglobin, and led him to conclude that the loss of an electron by the iron atom upon transformation of hemoglobin to methemoglobin enabled it to form an ionic compound with cyanide ion, as mentioned. It is clear however, from the experiments here described, that cyanide does form a compound with the ferrous forms of hemoglobin, giving cyanhemoglobin. The present state of our knowledge of the structure of the hemoglobin molecule does not afford any very satisfactory explanation of the nature of this union.

Conant (1933) has suggested that in hemoglobin the six coördinate positions available around the iron atoms are occupied by the four pyrrole nitrogens and two groups of the globin. One of the latter is supposed to be an un-ionized group, and to give way to a molecule of oxygen to form oxyhemoglobin. (Since there are actually four heme groups in the hemoglobin molecule, it is of course possible that intermediate stages of oxygenation may occur having 1, 2, 3, or finally 4 oxygen molecules thus taken up.) This theory leaves no coördinate position available for either cya-

nide ion or HCN without displacing some essential bond in the oxyhemoglobin molecule, such as globin or pyrrole group, or oxygen. Cook's experiments, referred to above, seem to exclude the possibility that any oxygen is displaced, and the separation of globin from heme, or the displacement of one or more pyrrole groups from the coördinate positions would presumably alter the absorption spectrum, a result which is not in accord with the experiments. The structure of cyanhemoglobin and cyanmethemoglobin must at present be considered to be unknown.

SUMMARY. It has been shown with the doses described in these experiments that:

1. KCN stabilizes the color of oxyhemoglobin by forming cyanhemoglobin. This substance has a spectrum identical with that of oxyhemoglobin.

2. No methemoglobin is formed in the blood stream or in defibrinated blood *in vitro* when methylene blue is used either with or without KCN.

3. The presence of reducing agents including glucose in the blood stream prevents the formation of methemoglobin.

4. $NaNO_2$ produces methemoglobin when it is used in amounts sufficiently large to overcome the reducing capacity of the blood.

5. Crystallized products of hemoglobin give results differing from the above because of the absence of reducing substances.

6. It is not justifiable to use experiments with pure crystallized hemoglobin derivatives or large excesses of reagents or prolonged methods involving conditions not found *in vivo*, to explain results obtained in cyanide and carbon monoxide poisoning and its therapy.

7. Possible theoretical considerations are discussed.

CONCLUSIONS

Cyanide attaches to either ferrous or ferric iron in hemoglobin derivatives forming either cyanhemoglobin (ferrous) or cyanmethemoglobin (ferric), depending upon the conditions of the experiment. In the blood stream with the doses described in these experiments, only cyanhemoglobin is formed.

Since no methemoglobin is formed by methylene blue in the blood stream with the doses used, it is again shown that the mode of action of methylene blue is as a catalyst, rather than as a producer of methemoglobin. Its use is again urged in both cyanide and carbon monoxide poisoning.

It is also again suggested that injections of glucose be used clinically in cases where it is desirable to reconvert methemoglobin to oxyhemoglobin in the blood stream.

Acknowledgments. My thanks are due the Board of Research of the University of California, and the State and Federal Emergency Relief Administrations for assistance in conducting these experiments. The

assistance of Miss Virginia Condit is also acknowledged. The assistance of Prof. J. H. Hildebrand of the Department of Chemistry of this University in formulating the theory is hereby gratefully acknowledged.

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THE AFFERENT FUNCTIONS OF NON-MYELINATED OR *C* FIBERS

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In a previous publication we have expressed the opinion that the non-myelinated fibers of the dorsal roots of vertebrate nerves, having their cells of origin in the dorsal root ganglia (Bishop, Heinbecker and O'Leary, 1933), and including similar vagus fibers having their cells of origin in the nodose, (Heinbecker and O'Leary, 1933) were not sensory in function, but motor. This conclusion was based on experiments under ether anesthesia, where the nerves were stimulated peripherally, their action currents led from electrodes proximal to the body, and all fibers except the non-myelinated were blocked between stimulus and lead by pressure. Under these conditions we obtained no reflex effects indicative of sensory response after block of *B* fibers, in the presence of a *C* action current response in the nerve itself, while before block the stimulation of *B* fibers only, by a weaker stimulus, gave a typical response indicative of sensory activity. On the other hand, stimulation of the non-myelinated fibers of the vagus, after cutting above the nodose and allowing time for degeneration, gave a peripheral motor response of bronchial and intestinal musculature, but not of the heart or of striated muscles innervated by the normal vagus. Further, the non-myelinated fibers are the elements in the dorsal roots which cause vasodilatation when stimulated toward the periphery.

More recently, Clark, Hughes and Gasser (1935) have reported positive reflex effects from similar experiments on the saphenous nerve after block by pressure, using dial anesthesia. Even without pressure block, they have observed an increase of effect of *C* fiber stimulation over that of *B* fiber stimulation, at a stage of anesthesia deep enough to reduce the *B* response to a relatively low value. This indicates a differential anesthetic effect on the central connections of *B* and *C* fibers, by dial as compared with ether. We have therefore repeated our former experiments, using both ether and dial separately, and in various combinations of the two, and confirm their general findings that *C* fibers can in fact give sensory and reflex responses on

[1] Assisted by a grant-in-aid from the Rockefeller Foundation for Research in Neurophysiology.

central stimulation; and that while dial depresses the response to *myelinated* fibers leaving a predominating effect of the *non-myelinated*, ether is differential in the reverse order, as compared to the non-anesthetized animal. We have further repeated our former experiments, but using dial, on stimulation of the peripheral stump of the vagus after degeneration of all fibers with cells of origin within the medulla. We again obtain what are definitely motor effects from the intestine, consisting either of augmentation of rhythmic contractions, or inhibition of rhythmic activity already present, depending on the condition of the animal. The responses are of the same order of magnitude on stimulation of the operated nerve as on stimulation of the normal nerve on the opposite side. The latter results will be reported separately in more detail. The present paper will deal with the differences in responses to stimulation of myelinated and of non-myelinated fibers under ether and under dial and under local anesthesia.

Two observations are pertinent to what follows. First, if the lethal dose of ether is determined for an animal (cat), then after partial recovery from ether, a full surgical anesthetic dose of dial is given intravenously (50 mgm./kgm.), and ether again administered, the lethal dose of ether, superimposed on the dial, is from 80 to 90 per cent of that before dial, as determined by blood concentration of ether at the time of stoppage of respiration. Since failure of the respiration under ether alone is immediately preceded by shallow but rapid respiration, whereas failure under dial alone is preceded by deep but extremely slow respiration of the apneustic type, these facts suggest opposite, even antagonistic effects on the respiratory center of these two agents; or more probably, *differential* effect on compensating or antagonistic elements of a complex respiratory mechanism.

The second observation has to do with the responses of peripheral nerve to stimuli strong enough to activate non-myelinated fibers (Bishop, and Heinbecker, 1935). A single shock of *C* strength in a normal unblocked nerve causes first a single volley from all the fibers, myelinated and non-myelinated. This may be followed by a random and repetitive discharge of the more irritable myelinated fibers, of very low amplitude but lasting over a large fraction of a second. Two shocks at $\frac{1}{10}$ second intervals sum in their persistent effects, both in the frequency of the following random discharge and in its duration. Several such shocks may give a response lasting over two seconds. Since the reflex responses of *B* and of *C* fibers are not readily distinguishable, reliable data on the effect of *C* stimulation can only be obtained by complete block of all fibers except the non-myelinated. A greater effect of stimulation of *B* plus *C* fibers, over the effect of stimulation of *B* alone, is equivocal, since it may be due either to *C* responses, or to a repetition of *B* responses, or to both together.

1. *A method for reversible block, differential between myelinated and non-myelinated fibers, and between large fibers and small.* Block by pressure

inactivates myelinated fibers before non-myelinated, but we have not found the method completely differential in cats, some *C* fibers being blocked before all the *B* fibers. It more completely separates the functioning of these groups in the saphenous of dogs and rabbits. It is time-consuming, allowing the status of the animal to change, and complicates the circuit when leading from the nerve beyond the block. Instantaneous induction of block, with speed of recovery from block depending only on the intensity of the blocking agent, can be obtained by means of alternating current, or quite conveniently by a brief series of strong make and break shocks from an induction coil. The preparation is arranged with three sets of electrodes, one at the end of the nerve, one next the body, and one between (fig. 1). The middle electrode pair connects to a Harvard inductorium

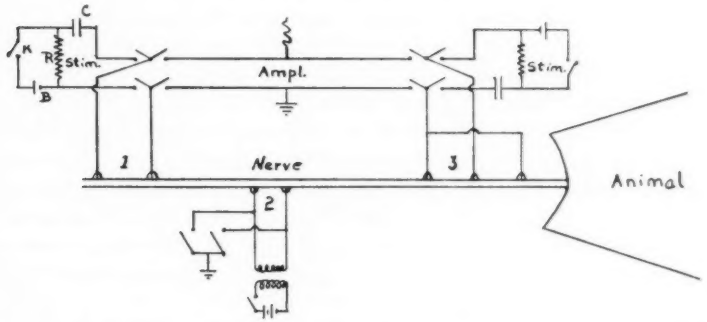

Fig. 1. Arrangement of nerve on electrodes for recording responses to *C* fibers only after block of *B* fibers by strong induction shocks. 1 and 3, stimulating and lead electrodes, interchangeable by double throw switches above. 2, blocking electrodes. *C*, variable condenser whose charge stimulates. *B*, tapped battery, *R* high resistance which permits slow discharge of condenser between stimuli, *K*, key on rotating interruptor.

with metal core, the secondary placed at about 6 on the scale for the average cat saphenous, the primary activated by two dry cells in series. Both poles of the secondary can be grounded by a double-point single throw switch, when this circuit forms a suitable ground contact between stimulus and lead. The two latter are connected to the center poles of double pole, double throw switches, one pair each of whose selector terminals leads to a stimulating apparatus, the other to the recording mechanism. The electrode set proximal to the body is suitably tripolar, the center pole leading to cathode or to amplifier grid, the outer poles to anode or ground. The record led from it will be triphasic, and 1 cm. distance between center and either outside pole is advisable to obtain a suitable amplitude of record. Since the current density at either anode during stimulation is only one-

half that at the central cathode, the danger of spread of stimulating current to the body is minimized, and *B* fibers are not blocked at these anodes by strengths of shocks required for eliciting even a maximal *C* response. The escape distortion is also minimized.

A brief contact of the induction coil primary switch, with the secondary ground switch momentarily opened, blocks the nerve fibers in the order of their thresholds, weaker shocks blocking only the lower threshold fibers. A fraction of a second contact will at suitable strengths block all but non-myelinated fibers without measurably reducing the *C* wave; they will remain blocked from a few seconds to a few minutes, depending on strength of shocks applied, and recover in the order of size, the slowest fibers first. As the blocking is repeated, it persists longer, or may be produced with weaker shocks. Still stronger shocks depress the non-myelinated fibers also, which however recover promptly, leaving a still longer interval (up to 15 minutes), when the *C* wave is maximal and all other potentials absent. During these intervals shocks of *C* strength are applied at the distal electrodes, and the *C* responses which alone reach the animal are recorded at the proximal electrodes; or shocks of *B* strength may be applied at the proximal electrodes, either alone or added to stimuli of *C* strength at the distal electrodes. By this means one may obtain the addition of *B* and *C* fiber responses at known frequencies of shocks, without the complication mentioned above, that strong shocks applied to an unblocked nerve may set up in myelinated fibers a random discharge of uncontrollable magnitude, frequency and duration.

If instead of a short series of strong shocks, a longer series of weaker shocks is employed, the myelinated fibers may again be differentially blocked, but in this case the *A* fibers may recover before the *B*, or at weaker strengths the *B* alone are eliminated. The details of the differentiation between *A* and *B* fibers, being of no further consequence here, will be reported more fully at another time. In all the experiments reported below the results are derived from nerves whose myelinated fibers were blocked differentially by strong shocks.

2. *Responses to saphenous nerve stimulation under ether, under dial, and under both.* In an initial series of 8 animals under ether anesthesia, 7 gave no response to *C* fiber stimulation, one gave a slight response. Anesthesia was fairly deep, but not sufficient to prevent responses to *B* fiber stimulation. In a following series of 20 animals, including cats, rabbits and dogs, under dial at various depths of anesthesia, we have never failed to obtain a clearly demonstrable response to *C* stimulation, consisting of skeletal motor, vasomotor and respiratory reflexes, and vocalization if anesthesia was not deep. In subsequent experiments under ether we have obtained *C* effects, particularly on blood pressure, which persists longest under ether, but responses to maximal *C* stimulation at a given frequency have disappeared

before responses to maximal *B* stimulation of the same frequency, as anesthesia was increased in depth. Central stimulation of the vagus may also give vasomotor responses under ether, and occasionally a slight respiratory increase, but the *B* effects are much the more prominent. Since *B* fibers can be stimulated at much higher frequency than can *C*, and presumably respond at higher frequency in their normal functioning in the body, and since the effect of *B* stimulation in these nerves at a given frequency is much greater, even in unanesthetized animals, than is the stimulation of *C* fibers, in spite of the greater number of the latter (Douglas, Davenport, Heinbecker and Bishop, 1934), the relative effectiveness[2] in causing responses of what may be termed provisionally a *B* reflex mechanism is very much greater than is that of the *C* mechanism. Fiber for fiber, the larger fiber gives the larger reflex (and presumably sensory) response per fiber impulse, as has been found for myelinated fibers of different sizes elsewhere (Bishop, Heinbecker and O'Leary, 1934).

Under light dial anesthesia ("hypnotic dose"), from which the animal is easily aroused by nerve stimulation, responses to both *B* and *C* stimuli seem to be even more persistent than under no general anesthetic at all (figs. 2 and 3). These animals were prepared under local intracutaneous anesthetic or under nitrous oxide, plus a little dial, to obviate any persistent effects of ether, or to avoid a heavy dose of dial that could not be dissipated within a reasonable time. The nerves could be blocked under these conditions by a series of strong shocks of such brevity that the equilibrium of the experiment was not seriously disturbed; longer durations of weaker shocks were not so well tolerated. There is some reason to believe that this hyperactivity observed under dial is psychic rather than simply reflex; a conscious dog may be quieted promptly after experimental excitement, but not so readily under light dial. The situation is perhaps similar to that of a person awakened violently from normal sleep.

A number of experiments were conducted on dogs, anesthetized initially with somewhat less than the usual anesthetic dose of dial. Responses to a given number and frequency of stimulation of *B* and *C* fibers separately were then recorded, and the anesthetic increased if necessary by intravenous injection until sufficient had been administered to significantly depress the *B* responses differentially as compared to *C*. Ether was then administered by tracheal cannula, until the previous response to *C* stimulation was

[2] In the saphenous nerve of the cat counted by these authors there were 1040 fibers above 6 mu., 1208 below 6 mu., myelinated and 6790 non-myelinated, of which latter 10 to 20 per cent would have been sympathetic fibers. Our counts on two normal skin nerves from human biopsies give respectively 105, 398, 863, above and below 6.4 mu. and non-myelinated, and 284, 851 and 1154. Since myelinated pain fibers are below 6.4 mu. in diameter, there are in general more non-myelinated fibers from the dorsal roots than myelinated of the pain size range in such sensory nerves.

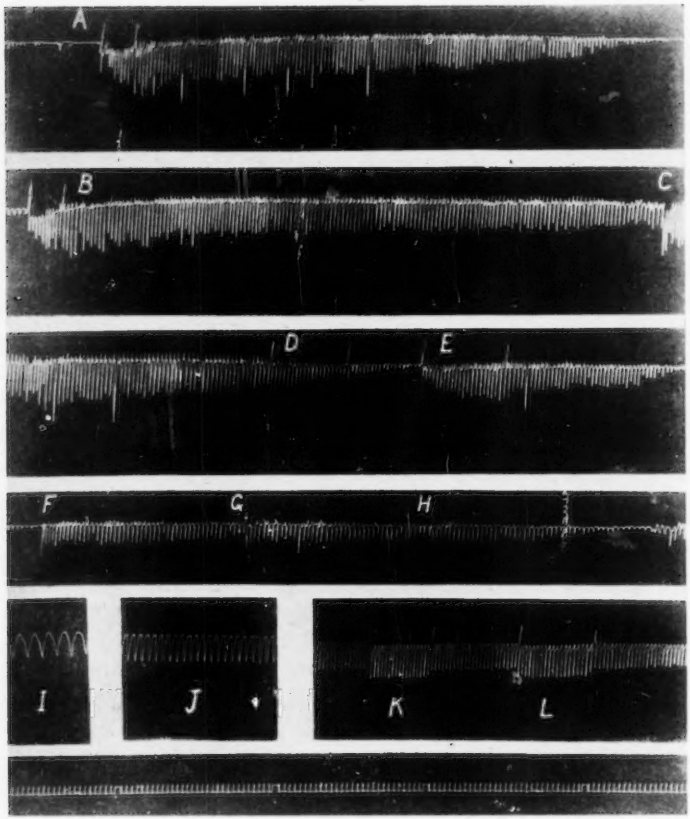

Fig. 2. 1/28/35. Dog under light dial anesthesia, ether added later. *A*, the last of 3 successive practically identical records to stimulation of *A* and *B* fibers only, saphenous nerve, 7 seconds at 6/second. Each response proceeds to apnea. Dial only. *B*, the same stimulation to *B* plus *C* fibers, 2nd of two responses. *C*, nerve blocked by strong faradic stimulation, record continuous with next strip. *D*, test for response to *B* fiber stimulus as in *A*, but twice the number, 14 seconds. *E*, stimulus of *C* strength, *B* fibers blocked, *C* wave as recorded below block is full amplitude. First of 3 identical records. *F*, *G*, *H*, *B* fibers blocked, 3 separate single crushes near end of nerve. *I*, respiration under dial alone, animal quiet. *J*, respiration after just enough ether to prevent occasional restlessness under dial alone. *K*, respiration under deeper ether, with stimulation of *A* and *B* fibers only, recovered from previous block. *L*, stimulation of *A*, *B* and *C* fibers, no increase over *K* under deep ether, no writhing movements of animal, but twitches of muscle of homolateral limb at each stimulus *K* and *L*. Time in seconds.

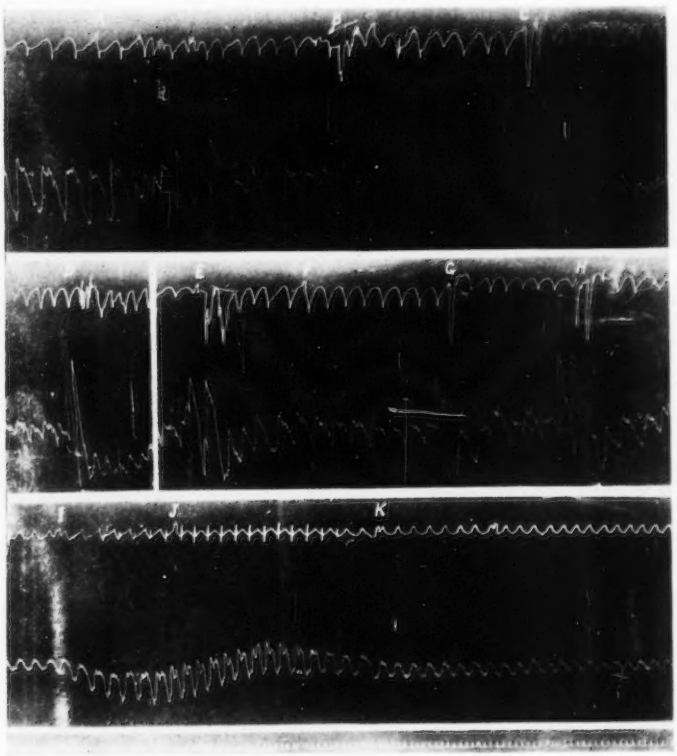

Fig. 3. 2/6/35. Dog, nerve dissected under local intradermal procaine anesthesia, respiration from tambour pneumograph, blood pressure 120 mm. Hg from femoral. *A*, no general anesthetic, stimulation of *A* fibers only, slight respiratory effect, 25 stimuli at 3/second. *B*, maximum *B* fiber stimulation, 25 stimuli, at 3/second. *C*, 15 stimuli to *B* fibers at 5/second. Record continuous with *D*, record of block of all *A* and *B* fibers by short period of strong faradization. After 1.5 minutes, *E*, 15 stimuli 3/second maximal for *C* fibers, *B* still blocked as shown by *F*, 15 stimuli at *B* strength. *G*, 15 stimuli at 5/second, *C* maximal, *B* blocked; and *H*, 25 stimuli, ditto. Dial was then given intravenously until the *B* response was materially decreased, *C* response still present, then ether was given. *I*, *B* recovered to $\frac{1}{3}$ its size before block, 15 stimuli at 3/second, respiration increases and animal yelps. During this response very brief faradization at *J* again blocked *B* fibers without noticeable reflex effect. *K*, 75 stimuli at 3/second. *C* wave maximal, causes slight if any response, *C* response blocked differentially to *B* by ether. Time in seconds.

prevented (fig. 3). Responses to *B* stimuli were still present. It appears as if these two reflex pathways were quite distinct up to the region in the cord where they find a common pathway, and that to low concentration of anesthetic, the *B* pathway is selectively blocked by dial, and the *C* by ether; higher concentrations either affect a common pathway, or affect both pathways. The effect of ether alone is illustrated in figure 4.

The behavior of animals under double anesthesia is characteristic. Under light dial the animal may be perfectly quiet until placed in position

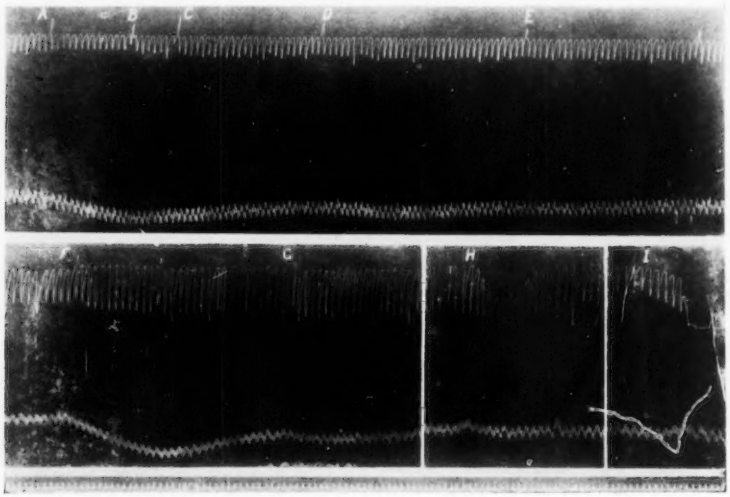

Fig. 4. 2/18/35. Dog, ether anesthesia, differential effects on *B* and *C* fiber responses, blood pressure from carotid. *A*, deep anesthesia, maximal *B* fiber stimulation, *B* wave ½ amplitude of normal after previous block, 5 stimuli in one second. *B*, *B* fibers blocked. *C*, 5 stimuli, *C* wave maximal, trace of *B* wave remains. *D*, *B* fibers blocked again, *E*, 5 stimuli, *C* wave maximal, no *B* wave. Animal allowed to come out of ether. *F*, *B* fibers nearly completely recovered, 5 stimuli *B* strength. *G*, *B* fibers blocked. *H*, *C* fibers only stimulated maximally, anesthesia becoming continually lighter. *I* repeated *H*, animal struggles. Time in seconds.

on the animal board, after which the tail lashes, the tongue is extruded and moves continuously (particularly in cats), periodic writhing movements occur, etc. A very slight administration of ether on a sponge, much less than would be required of ether alone, quiets the animal completely. At this stage, then, and in this respect, ether and dial sum in their anesthetic actions most effectively. The same applies to the effects of stimulation; a little ether converts the exaggerated responses under light dial to those characteristic of a much greater amount of ether, and the effect is per-

sistent for a surprisingly long time after ether administration is stopped. On the other hand, when the respiration under even a little dial is characteristically slowed, the addition of a little ether quickens the respiration (fig. 2-I, J). This may occur even under deep dial anesthesia, when no respiratory reflexes are obtained from nerve stimulation, and is therefore not due to stimulation of the respiratory endings in the walls of air passages.

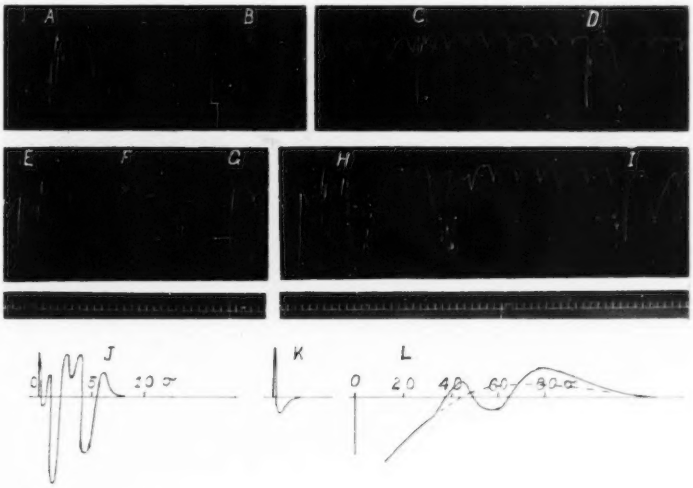

Fig. 5. 2/4/35. Dog, local anesthesia over course of saphenous nerve, no general anesthesia. *A*, 5 stimuli of *B* strength, *B*, *B* fibers blocked. One minute elapsed time testing for *B* wave, to *C*, 5 stimuli somewhat submaximal for *C* fibers, *D*, maximal *C* wave, 5 stimuli. The second deep respiratory wave is a sigh. *E*, *B* fiber block. *F* stimulation at *B* strength, and *G*, at *C* strength. After 2 minutes, *H*, 5 stimuli over electrodes proximal to body, *B* strength, *I*, 5 *C* stimuli at end of nerve, still blocked midway. Time in seconds.

J, trace of oscillograph contact print, *A* and *B* waves, *K*, shock only at *B* strength after block of *B* fibers, *L*, *C* wave at end of experiment, presumptive base line of shock distortion in dash line. Amplification 150 mm/mv. $\frac{2}{3}$ size.

3. *Responses to nerve stimulation after nitrous oxide anesthesia, and under local anesthesia.* Although ether is eliminated rapidly through the lungs, it is probably selectively retained by nervous tissue, and the experience of human patients would indicate that its effects are more persistent than its volatile character might suggest. We presume that our previous failure to obtain reflex effects from *C* fiber stimulation in dogs decerebrated under ether may have been due to incomplete elimination of the ether. To check this, and to ascertain whether dial was inducing or exaggerating *C*

responses as compared to the normal animal, dogs were operated under nitrous oxide-oxygen anesthesia, and under intradermal anesthesia alone over the course of the saphenous nerve. Otherwise the experiments were identical to the previous ones. Effects of stimulation of *C* fibers alone were unequivocally present (figs. 3, 5, 6, 7). With a minimal number of maxi-

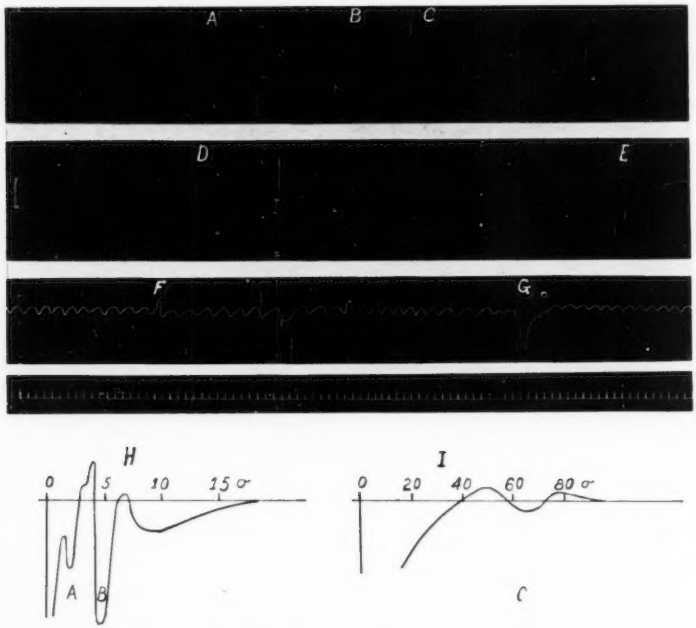

Fig. 6. 2/4/35. Dog, nitrous oxide during operation, then no anesthesia *A*, *B*, 2 short faradizations to block *B* fibers. *C*, five *B* stimuli in 1 second, repeated, *D*, strip continuous with above, 5 *C* stimuli, followed by repeated periods of *B* stimuli as *B* fibers return from block until response at *E* when *B* wave was ¼ normal size. Later in experiment, dog less responsive, *F*, 125 stimuli at *C* maximal strength, slowing of respiration followed by quickening after stimulation. *G*, 5 stimuli *B* strength, *B* wave ¼ size, respiration speeds up. Time in seconds. *H*, oscillograph record somewhat later, *B* wave nearly maximal, *A* just appearing. *I*, *C* wave photographed during *F* above. Amplification 220 mm/mv. reduced ½.

mal stimuli for *B* or *C* fiber groups separated by block of *B* fibers, the results varied from animal to animal depending on the animal's "temperament" and the effectiveness of the attendant in keeping it quiet by talking and kindly manipulation. A given minimal effect, however, on respiration or on body movement or other signs of distress, was obtained by definitely fewer stimuli to *B* fibers than to *C*, in a given animal, and a longer latency

of response was observed to *C* stimulation than to *B*, or a given number of stimuli of *B* fibers gave greater effect than the same number of *C*. Reflex effects in the homolateral limb as indicated by action currents led from muscles exposed in dissecting the nerve showed the same differences (see below) as were obtained from animals under deeper anesthesia.

4. *Comparison of the character of response to B fiber and to C fiber stimulation.* In general, reflex responses to *C* fiber stimulation under dial or after local anesthesia only, show a longer latency, a less effect per impulse per fiber, and a smoother summation of effects of stimuli of low frequency, than

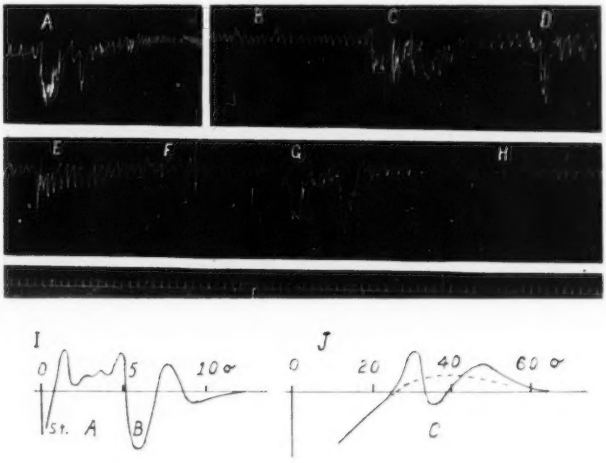

Fig. 7. 2/6/35. Dog, nitrous oxide during operation. *A*, 10 shocks, 6/second *B* strength. *B* fibers then blocked. *B*, 30 stimuli at 6/second test for *B* response. *C*, 30 stimuli *C* strength. *D*, repetition of *B*, but 10 per cent of *B* wave has returned. *E*, *B* fibers blocked, *F*, *B* test as above, *G*, 30 stimuli *C* strength, *H*, same, *B* strength. Time in seconds. *I*, oscillograph record of *B* wave return after block, nearly maximal, with trace of *A*, much delayed. *J*, *C* wave after *B* block, 170 mm/mv. reduced ½.

obtains for responses to *B* fiber stimulation. We have made some preliminary effort to find whether *different* reflexes were called forth by these two groups of fibers in the same nerve. In some cases the opposite is true as judged by our present criteria of what a unit reflex is. Both mechanisms increase the frequency and depth of respiration, on stimulation of either saphenous or vagus. Both give the initial decrease or paralysis of respiration at the initiation of strong stimulation. We have not usually observed a slowing of respiration due to *C* fiber stimulation alone, such as may be obtained from myelinated fiber activation under selective conditions of intensity and frequency of stimulation. Both mechanisms may either

raise or lower the systemic blood pressure, depending again on the condition of the experiment and the state of the animal. We do not feel that this is significant until we know in what manner local reflexes, that is, vasoconstriction or dilatation in local regions corresponding to a circumscribed reflex pathway, contribute to the general systemic effect. Stimulation of either group of fibers may dilate the pupil. General body movements are produced by either; under dial, these occur only after *C* stimulation if the frequency of stimulation is slow, but apparently identical responses can be obtained due to *B* stimuli of higher frequency. Under ether the order of occurrence of these movements is reversed. Without anesthetic they accompany all stimuli above threshold ones, and are presumably more complex in the conscious animal than simple reflexes.

In the responses of the skeletal muscles of the homolateral limb (and presumably in other muscles where we have not yet investigated them), the reflexes to *B* and *C* fiber stimulation differ so characteristically as to justify the inference that there are separate *B* and *C* reflex mechanisms, which may employ the same motor mechanisms in the periphery for their final expression, but whose courses through the spinal cord to reach these final pathways may be differentially modified.

The response to a single *B* stimulus of the homolateral muscle in a lightly or non-anesthetized animal is essentially a twitch, or a brief contraction in which several twitches are summed, of the character of the knee-jerk. The first response to a series of maximal *B* stimuli of low frequency may be greater than successive responses, or the first few may increase, the following responses decrease in value, the responses being discrete as indicated by the action currents of the muscle. At higher frequency or sufficiently long duration of low frequency, a summated tonic or clonic response follows, involving the limb as a whole, with twitches superimposed for each stimulus. Rapid stimulation under deeper anesthesia may result in fusion of such twitches to a smooth tetanus without widespread involvement of the limb as a whole in more complex movements. The action currents of the muscle correspond, except that the periodic discharge timed with the stimulation may be seen to be complicated, after the more severe stimuli, by a random or scattering discharge of lower amplitude. The latent period to a single shock is brief, of the order of that of the knee-jerk.

The response to a single *C* stimulus uncomplicated by *B* responses has a much longer latent period, when it causes a response at all. Usually several stimuli are required. They are summed smoothly even at low frequencies, no twitch-like responses occur, but a tonic contraction takes place with random discharge of muscle action currents, the contraction building up in tension with successive stimuli. This passes over into either an extensor spasm involving both limbs, or into writhing or struggling movements. The final result of either *B* or *C* responses is thus similar

except that the *C* response lacks completely the specific twitches characteristic of the *B*.

5. *Responses to stimulation of nerve to muscle and of sympathetic chain.* One experiment was performed, under dial, in which the dog was eviscerated and the abdominal sympathetic chain stimulated. At *B* strength, each stimulus called forth a twitch reflex of the abdominal wall muscles. No muscle reflex effect could be observed on stimulation of *C* fibers following block of *B*, although the *C* wave was higher in amplitude and much larger in total area than the *B*. Presumably most of these fibers were sympathetic efferents. Small responses of blood pressure and respiration were obtained from *B* stimulation, with a possible slight increase as *C* stimuli were added. Evisceration had removed in this case the structures in which a response might have been expected to take place.

In one animal under dial, the nerve to the medial head of the gastrocnemius was stimulated. In this preparation, in contrast to the sympathetic, the respiratory response to *C* fiber stimulation alone was greater than that to *B*; after a given number of stimuli, just sufficient to cause a howl on *C* stimulation, *B* stimulation caused only weaker struggling. Administration of ether just sufficient to stop restlessness and continued whining under dial alone abolished the respiratory response to *C* stimulation and reduced the response to *B* stimulation materially. The *C* wave in the muscle nerve is much smaller than in the saphenous, but a *B* wave is absent in the sensory component of the muscle nerve, the *B* fibers being represented as a decremental prolongation only of the *A* elevation (O'Leary, Heinbecker and Bishop, 1935). Stimulation of *B* fibers of this muscle nerve however in conscious human subjects at operation under local anesthesia (unpublished) causes sharp pressure pain referred to the muscle, which is accurately differentiated from the pain due to similar stimuli to adjacent skin nerves.

6. *Are non-myelinated fibers ambivalent?* We have previously reported what seemed to be reflex vasodilator responses, over the dorsal roots in sympathectomized animals (Bishop, Heinbecker and O'Leary, 1933) confirming Bayliss (1901) and Fofanow and Tschalussow (1913), and assigning these effects to the non-myelinated fibers of the dorsal roots. We had given a like significance to the apparently motor effects obtained by stimulation of the peripheral stump of the vagus after cutting above the nodose and degeneration of all known motor fibers of medullar origin. Since, however, the non-myelinated fibers also give afferent effects, the question arises whether to designate their efferent action as antidromic, or simply as motor. In the latter case the implication would be, either that two groups of these fibers exist, one group sensory and one motor, or that the same fiber normally in the bodily activity was capable of being activated at either end, and of conducting an impulse in either direction.

We cannot choose between these alternatives at present, but two con-

siderations may be of significance here. First, the only afferent fiber group to which an "antidromic" or "motor" function can be assigned at present is the group of non-myelinated fibers of the dorsal roots, and homologous fibers in the vagus. Second, Speidel (1934) has found that in nerve twig regeneration the non-myelinated fibers, anastomosing with other non-myelinated fibers from other twigs, may not degenerate distal to a cut, and that these fibers contribute to growing myelinated fiber cell elements essential to the myelination of the latter. Both these facts, of anastomoses, and of the relation to myelinated fibers, attest the more primitive character of non-myelinated axons. The possibility that peripherally at least, there may be involved transmission from fiber to fiber through a terminal network remains to be tested. The peculiarities involved in the functioning of these fibers indicate accessibility to stimulation at their central ends as well as peripherally. Under such a situation the axon reflex supposed to account for certain local phenomena in skin stimulation would also serve a central reflex mechanism for vasodilatation in the skin, and motor activity in the intestine might be brought about similarly.

SUMMARY

1. The functions of non-myelinated fibers have been reinvestigated under different anesthetics, and after differential and reversible block of myelinated fibers by alternating current.

2. Dial as an anesthetic reduces the reflex effect of the stimulation of myelinated fibers differentially as compared to non-myelinated fibers, (confirming Clark, Hughes and Gasser) and ether acts differentially in the reverse order.

3. NO_2–O_2 anesthesia seems to have a less differential effect than the other two anesthetics employed; the actions of all three have been checked against the normal response obtained after local anesthesia only at the site of the nerve dissection.

4. The reflex effects of stimulation of myelinated and of non-myelinated fibers are in some respects different. Certain muscle responses to myelinated fiber stimulation consist of a succession of twitches on repeated stimulation, even the first stimulus being effective, which are summed to a smooth tetanus only when the frequency of stimulation is high. Responses of the same muscles to non-myelinated fiber stimulation usually require more than one stimulus, and are smoothly summed at slow rates of stimulation. The action currents of the muscle show a random discharge, in contrast to the succession of volleys characteristic of the responses to myelinated fiber stimulation of the same frequency.

5. The reflex responses to non-myelinated fiber stimulation in the saphenous, the nerve to the medial head of the gastrocnemius, the sympathetic trunk and the vagus, are similar, with respect to respiration, blood

pressure, and pupillary dilatation, and except for localization, with respect to muscle responses. They undoubtedly are capable of producing pain in all these nerves, the degree apparently depending chiefly on the number of fibers available in each nerve. Less effect is produced per impulse per fiber than for myelinated fibers.

6. Since in the spinal dorsal roots and in the vagus degenerated above the nodose ganglion stimulation toward the periphery results in efferent or motor effects, but only the stimulation of non-myelinated fibers has this result, it is considered possible that these fibers have a central motor connection and a peripheral motor ending, as well as the sensory connection, "Antidromic" effects and "axon reflexes" in the periphery would on this basis involve their activity as efferent pathways.

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ANTIANEMIC TREATMENT IN EXPERIMENTAL POLYCYTHEMIA[1]

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The therapeutic value of liver in anemia is widely known. It has been suggested in a preliminary report (Hanson and De Savitsch, 1934) that the rôle of the liver may be more general than is indicated by its action in anemia, that the liver may function to reëstablish the normal number of erythrocytes in the blood whether these be too low or too high. If the liver does act as a regulator, then its administration should in some degree be successful therapeutically in conditions of polycythemia.

To test this hypothesis, a sustained polycythemia was produced in experimental animals, which were then treated with liver. Red cell counts at frequent intervals revealed the correctness of the assumption.

Among the various methods of producing polycythemia experimentally may be mentioned the administration of germanium dioxide (Hammett and others, 1922), injection of serum from an animal in which the erythroblastic reaction is taking place (Carnot and Deflandre, 1906), and feeding a diet low in ash content (Swanson and Smith, 1932). The agent most frequently used is cobalt, administered as the chloride, nitrate or glutamate salt. Mice, guinea pigs and frogs have been found sensitive to cobalt (Sutter, 1934), while dogs, rabbits and rats have been used most extensively.

The hematopoietic effect of cobalt was first noticed by the Waltners in 1929. These workers found that injecting or feeding cobaltous salts produced an acute polycythemia in rats. The following year the abnormally red and hyperplastic bone marrow characteristic of the condition was seen in cobalt-fed dogs (Mascherpa, 1930). Orten and his co-workers (1932) administered 0.5 mgm. of cobalt per day to rats on a milk diet supplemented by salts of copper and iron. This treatment increased the red blood cell count, the hemoglobin and cell volume, while the leucocyte value was not significantly altered. Further work (Orten and others, 1933a) showed that manganese added to the régime not only appeared to stabilize

[1] A preliminary report of this work was presented before the American Physiological Society on March 30, 1934.

the polycythemia, but also alleviated any toxic effects of long-continued administration of cobalt. This latter finding has been confirmed by Kleinberg (1934) on rabbits. Later workers (Beard and Andes, 1934) claim that it is not necessary to add copper to the diet.

EXPERIMENTAL WORK: METHODS. The present experiments have been carried out over a period of twenty months. White rats from the stock colony were weaned at the age of 21 days and distributed among experimental and control groups as evenly as possible as to sex and litter. The groups were kept in separate cages bedded in sawdust. At the age of four to five weeks, the stock diet was discontinued, and whole milk supplied ad libitum, supplemented with cod liver oil and yeast. This diet permitted maintenance of health and a continued growth below that of normal rats. At the same time each animal received daily 2.5 cc. of a solution containing cobaltous chloride, cupric sulfate, ferric chloride and manganese sulfate in such quantities that each dose contained 0.5 mgm. cobalt, 0.5 mgm. iron, 0.025 mgm. copper, and 1.0 mgm. manganese. It was found convenient to

TABLE 1

Erythrocyte counts in millions per cubic millimeter with and without ether anesthesia

YOUNG NORMAL FEMALES	ANIMAL 1	ANIMAL 2	ANIMAL 3	AVERAGE
Without anesthesia	5.95	7.72	7.42	7.03
With ether one hour later	7.80	7.40	6.55	7.25
YOUNG NORMAL MALES	**ANIMAL 4**	**ANIMAL 5**	**ANIMAL 6**	**AVERAGE**
With ether	9.25	10.07	7.47	8.93
Without anesthesia one hour later	9.37	9.77	7.57	8.90

administer the solution by stomach tube. A control group fed the normal stock diet was run simultaneously with each series of experiments.

After two to three weeks on the milk-mineral régime, preliminary erythrocyte counts were made to ascertain the degree of polycythemia. These counts were done every other day until three of the same magnitude were obtained. The usual diluting pipet and improved Neubauer counting chamber were washed and dried between counts, so that the same ones served throughout the entire series of experiments. Hayem's solution was the diluting fluid, and three squares of sixteen on one scale plus two on the other were counted. By taking squares in the same position each time, choice was eliminated.

Blood was taken directly by heart puncture under light ether anesthesia. Comparative counts on the same animals with and without ether showed no appreciable difference in the numbers of circulating erythrocytes. These figures are presented in table 1.

Treatment was carried out over a period of two weeks, during which time the cobalt administration was continued and the erythrocytes were counted every other day. In a few cases, reticulocyte counts were made. These were vitally stained with brilliant cresyl blue, and the smears stained with Wright's stain.

RESULTS. The results of all experiments carried out as controls are presented in the first part of table 2, and are graphically represented in figure 1.

Normal control animals showed an erythrocyte count which steadily increased from an average of approximately 7.5 million cells per cubic

TABLE 2

Average experimental and control erythrocyte counts in millions per cubic millimeter

NUMBER OF RATS	CONDITION AND DAILY TREATMENT	CONTROL PERIOD			DAYS AFTER INITIAL TREATMENT						
		I	II	III	2	4	6	8	10	12	14
12	Normal—untreated	7.98	7.81	7.82		8.55		8.12	8.29	8.82	8.42
8	Normal—liver extract	7.21	7.31	7.27		7.12		7.37		7.84	8.06
16	Polycythemic—saline	11.16	11.71	11.61	11.60	11.62	11.64	10.98	11.20	11.39	11.30
7	Polycythemic—kidney extract	10.27	10.86	10.93	10.60	11.29	10.80	11.03	10.56	11.24	10.43
4	Polycythemic—raw meat	10.88	11.15	10.88	11.24	11.41	10.96	11.08	11.20	10.51	11.42
23	Polycythemic—liver extract	10.90	11.44	11.86	10.98	10.64	9.93	10.67	11.39	11.85	12.01
12	Polycythemic—ventriculin	10.50	10.44	10.69	10.70	9.86	10.53*	9.81	10.18*	9.73	9.63
8	Polycythemic—raw liver	10.11	10.26	10.56	11.65	11.53	10.26	11.04*	10.14	10.24	9.80

* These figures represent experiments on reduced dosage.

millimeter to 8.5 million. This is a typical increase with age for rats (Donaldon, 1924). Intramuscular liver extract[2] injections in a group of eight normal animals produced no demonstrable effect upon the erythrocyte count, other than the normal increase with age mentioned above. This is contrary to the findings of Watkins (1928). Reticulocyte counts on two untreated normal animals were below 1 per cent.

Polycythemic control animals yielded preliminary values ranging from ten to thirteen million erythrocytes per cubic millimeter, with an average of

[2] The first experiments were performed with Lederle extract, which was unsatisfactory. We are greatly indebted to Eli Lilly and Company for a tested concentrated extract, which was used throughout the experiments reported at this time.

11.5 million, as compared to an average of 7.6 million for the normal controls. The counts increased with the length of time on the cobalt régime, as may be seen by comparing the three figures obtained for the preliminary control periods in all groups on table 2. Daily intramuscular injections of 0.25 cc. saline did not alter the count. The reticulocytes of two polycythemic control animals averaged 3.8 per cent.

Kidney extract[3] was tested on a group of seven animals to determine whether the response found with liver extract might be obtained with that of another tissue. Daily intramuscular injections of 0.25 cc. kidney extract caused toxic symptoms and fluctuation in the erythrocyte count which at

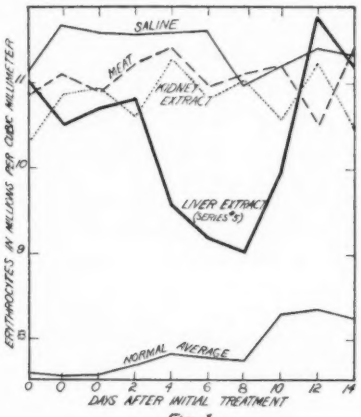

Fig. 1

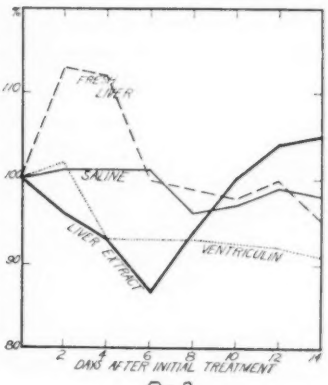

Fig. 2

Fig. 1. Erythrocyte counts of normal and polycythemic control rats, from data of table 2. Included for comparison is the curve of a typical group of four polycythemic animals receiving liver extract.

Fig. 2. Percentage variation in erythrocyte counts of treated and untreated polycythemic rats, taking the average of the control period counts as 100. Data from table 3.

no time fell below the initial polycythemic level for this group. By no means does this indicate that the anti-polycythemic action of the liver is specific, however, for the kidney extract was an alcoholic one, while that from the liver was aqueous.

The results with experimental groups are summarized in the second part of table 2. Table 3 and figure 2 present in percentages the variation from the control polycythemic level brought about by liver extract, ventriculin and fresh liver.

Liver extract was injected into the thigh muscles of a total of 23 poly-

[3] Furnished by Abbott Laboratories, North Chicago, Illinois.

cythemic rats. The daily dose of 0.25 cc. represented the material from approximately 8.3 grams of fresh whole liver. This treatment caused a brisk but temporary fall in the red cell count, which in six to eight days amounted to from 1½ to 2 million cells per cubic millimeter. At the lowest point, the average fall was to 87 per cent of the polycythemic control level. In a few cases the count fell to that of the normal controls. Among the animals tested, there were several found to be irresponsive to the treatment. This may be due to some absolutely refractory condition of the animals, or to a period of treatment too short to discover a slow response. The lowered erythrocyte count could not be maintained, the level rising again to an even higher value than before, in spite of continued administration of liver extract. Sometimes an increase preceded the fall, as may be seen from the curve of a typical group of four animals included in figure 1. Reticulocyte counts on two animals which received liver extract injections

TABLE 3

Percentage variation from control erythrocyte counts of normal and of treated polycythemic rats

CONDITION AND DAILY TREATMENT	CONTROL AVERAGE IN MILLIONS PER CMM. (= 100%)	DAYS AFTER INITIAL TREATMENT						
		2	4	6	8	10	12	14
Normal—untreated	7.57		103		102	109	110	109
Polycythemic—saline	11.49	101	101	101	96	97	99	98
Polycythemic—liver extract	11.40	96	93	87	94	100	104	105
Polycythemic—ventriculin	10.54	102	93	100*	93	97*	92	91
Polycythemic—raw liver	10.31	113	112	100	107*	98	100	95

* These figures represent experiments on reduced dosage, and for the sake of simplicity are not included on figure 2.

averaged 2.1 per cent. These counts were made when the treatment was having most effect, as shown by the erythrocyte level, and yield a value not quite half as great as that of the untreated polycythemic controls, but still higher than normal.

Since the response to liver extract was so spectacular, we were interested in seeing whether other antianemic agents would be successful. With this in view, 2.5 grams of hog gastric mucosa preparation[4] were added to the milk-mineral régime of twelve polycythemic rats. The resulting fall in red blood cell values was not as great as that obtained with liver extract, but the lowered value was maintained throughout the experimental period. When the amount of ventriculin was decreased, as happened on the sixth and tenth days after the initial treatment (these figures are marked by asterisks on tables 2 and 3), the value rose again towards the polycythemic

[4] Parke, Davis and Company's "ventriculin."

control level. Doubling the amount of ventriculin did not appreciably alter the response. Reticulocyte counts on two particularly responsive animals averaged 2.0 per cent, a value almost identical with that obtained for the animals receiving liver extract.

Raw liver was given to eight polycythemic rats, each receiving approximately 8 grams of fresh tissue per day. This amount corresponds roughly to the material administered per day in the liver extract injections. This resulted in an immediate further rise of 13 per cent in the already high erythrocyte count. By the sixth day the count returned to the control polycythemic level, around which it remained for the rest of the period. It might be possible that some of the potency of the fresh liver tissue was destroyed during digestion, so that the amount administered per os was not comparable to that injected. Reticulocyte counts on two animals averaged 1.4 per cent, a figure which indicates that raw liver was more effective than the other forms of treatment in suppressing the numbers of circulating immature red blood cells.

By way of control, four polycythemic rats were fed approximately 8 grams of raw, lean meat. This caused fluctuations in the erythrocyte count, but resulted in no significant change.

Discussion. There can be no doubt that the increase in number of red blood cells brought about by cobalt is a true polycythemia, due to an increase in the rate of formation of new cells over the rate of destruction. That it is not a relative polycythemia, brought about by the concentration of erythrocytes through loss of plasma, is ruled out by the studies of Orten and others (1933b). These workers found that in chronic experimental polycythemia, there is an increase in the total blood volume, due to a rise in cell volume as well as to increased numbers of cells. The absence of large variations in the leucocyte count also eliminates the occurrence of a concentration of blood. The factor of mobilization of previously noncirculating cells has not been directly disproven, but the following considerations make it improbable. First, the macroscopically visible hyperplastic bone marrow, to which we have already referred, indicates increased activity on the part of the erythropoietic tissue. Secondly, the formation of new erythrocytes is indicated by our own increased reticulocyte counts of the untreated polycythemic control animals, and by the reticulocytes and dividing normoblasts found in the circulating blood of rabbits made polycythemic by cobalt (Klienberg, 1934).

The mechanism by which cobalt produces a true polycythemia is unknown, but there are several clues at present. The most direct evidence is that of Berwald and others (1934), who have found that cobalt sulfate is unable to produce polycythemia in splenectomized rats. A few informal studies of our own indicate that the milk and mineral régime does not produce microscopically visible changes in the liver. The fact remains

that in some direct or indirect manner, cobalt stimulates the formation of erythrocytes in the red bone marrow.

It becomes interesting to compare cobalt polycythemia with the condition of primary polycythemia in humans (erythremia, Vaquez-Osler syndrome, splenomegalic polycythemia, polycythemia rubra). First described by Vaquez in 1892, and elaborated upon by Osler (1903), this usually fatal disease is characterized by an increase in blood volume accompanied by vascular engorgement of all organs. The increased blood volume is due to increased numbers as well as size of the erythrocytes (Deleonardi, 1934), while the leucocytes and other formed elements may or may not be increased. This blood picture relates the human disease to experimental polycythemia in animals. First descriptions of the disease are very positive about the fact that the red marrow is the only tissue showing hyperplastic changes. Careful searching by later workers (Delannoy, 1924; Letulle and Yacoel, 1924), has revealed evidences of hematopoietic activity in the spleen. Hirschfeld (1925) declares that even in light cases can hemopoiesis be seen in the spleen, in addition to the usual changes in the red bone marrow.

The condition of primary polycythemia has been ascribed to many causes (see Weber and Bode, 1929), but most writers agree upon the truth of Vaquez' original contention that the hematopoietic tissues are primarily at fault. Today, however, it is necessary to include the spleen as a hematopoietic tissue, in view of the autopsy evidence.

The suggestion of an hormonal control of the blood-forming tissues is not a new one. The early work of Carnot and Deflandre (1906) suggested this possibility. These investigators found that a small loss of blood conferred upon rabbits' sera an activity strongly hematopoietic when injected into new rabbits.

Hormones from several sources have been tentatively suggested as controlling hematopoiesis.[5] On the basis of one clinical case, Morris (1933) presents "addisin," from the gastric mucosa, as a stimulator to the bone marrow. He found that his patient's polycythemia was made worse by intravenous injection of addisin, while lavage incidental to the treatment of duodenal ulcer brought a ten million erythrocyte count down to normal in six months. If addisin is comparable to or contained in ventriculin, then Morris' findings do not confirm our results with ventriculin in cobalt polycythemia. However, the difference in mode of administration may be a contributing factor.

Several writers have attributed the hormone to the liver. This hormone may play a stimulant or inhibitory rôle; if it stimulates the bone marrow, then polycythemia is held to be the result of hypersecretion; if it inhibits

[5] For a discussion of the occurrence of polycythemia in endocrine disturbances, see Gunther (1929).

the marrow, then insufficient secretion would remove a check upon erythrocyte formation. The former view is that of Hirschfeld (1925), who supports it with the fact that anemic patients have been benefited by administration of serum possessing hematopoietic qualities after previous blood-letting. The latter view is supported by our own findings with liver extract injections, and may be invoked to explain the results of others.

Phillips (1933) saved a polycythemic patient, after other measures had failed, by irradiation of the liver. He attributes the cure to stimulation of the liver to take over the blood-destroying activity of the removed spleen. It is not impossible, however, that irradiation should have stimulated the secretion of a hormone which checked the overactivity of the red bone marrow.

Stephan (1930) found that both spleen and liver extract given orally produced a prompt response in the few polycythemic patients on whom it was tried. Stephan explains this not by assuming a hormone from the liver, which is stored in the spleen, but by assigning the origin of the material to the suprarenal cortex. He presents experiments with adrenalin-free cortin which brought about a slight drop in erythrocyte count in a few hours. However, the experiments seem too few in number and the change in red cell count too small to be conclusive.

Although we hesitate to add to the legions of dubious hormones already postulated for the liver, the balance of evidence indicates an hormonal control by the liver inhibiting the red bone marrow. The fact that we were not able to detect microscopic changes in the secretive elements of the liver, and that liver changes in the Vaquez-Osler syndrome have not been found, does not obviate the possibility that polycythemia is the result of a decrease in the amount of inhibitive liver hormone. On the other hand, it may be that the organism's need for the hormone has increased, and that there has been no compensation for the increased need, so that the normal supply becomes inadequate. The postulation that the liver and spleen act as a storage place for a hormone whose origin is elsewhere seems unnecessary.

Our results with ventriculin indicate that this substance contains the hormone or stimulates the liver to its production. The fact that the lowered erythrocyte count was maintained throughout the experiment by ventriculin leads us to believe that further experimentation with this substance would be more successful than that carried out along other lines. The temporary effect of liver extract, with a return to even higher figures, may indicate that we are dealing with two substances, of which the inhibitive one soon stimulates the development of an immunity to itself. Such a supposition makes possible an explanation of the absence of lowering effect of fresh whole liver per os, as the inhibitive substance may succumb to digestive processes. The hypothetical character of the explanations which we have assayed makes further research imperative.

We are especially grateful for the encouragement and assistance which Prof. Anton J. Carlson has given this work.

SUMMARY

A true polycythemia has been produced in rats by the administration of a milk diet supplemented by salts of cobalt, iron, copper and manganese. Red cell counts of blood obtained by heart puncture revealed a polycythemia of from 10.5 to 13 million cells per cubic millimeter, in contrast to 7.5 to 8 million for the normal controls.

The high erythrocyte count has been promptly lowered to an average of 87 per cent of its initial level within six days by daily injections of 0.25 cc. concentrated liver extract. Controls receiving injections of saline or kidney extract maintained a count averaging 11.5 million. The fall in erythrocytes was only temporary, a return to above the initial level occurring although the liver extract was continued.

Administration of ventriculin brought about a more gradual and less pronounced decrease in erythrocytes, which was maintained throughout the experiment.

Feeding of fresh whole liver caused a temporary increase in the already high erythrocyte count, but no lowering occurred. Fresh lean meat produced no change.

Certain similarities between cobalt polycythemia in rats and primary polycythemia as it occurs in humans have been discussed, and some of the theories of direct or indirect hormonal control of the erythroplastic tissues have been presented. The evidence presented is interpreted by assuming a hormone which originates in the liver and exercises an inhibiting action upon hematopoiesis.

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PLASMA PROTEIN REGENERATION AFTER BLEEDING IN THE RAT

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The origin of the large amount of protein which enters the blood during and after perfusion in the rat (1) has been presumed to be in the tissue spaces or organ depots. In an effort to place this site of origin more certainly, the amount of protein regeneration in the blood after a single large bleeding was measured, and the influence of splenectomy, fasting, and injection of India ink on the regeneration noted.

METHOD. The method is a modification of that described in detail by the authors (1) in the determination of total plasma protein in rats. From one of the jugular veins a rat is bled of about 40 per cent of his total blood volume. This is called specimen I. For the succeeding 12 hours the rat is allowed water but no food; then the jugular vein is again opened and another large sample of blood taken and designated specimen II. Immediately a cannula is inserted in the other jugular vein and the remaining blood of the rat is washed out with normal saline solution and collected as specimen III.

To determine the rat's blood volume at the conclusion of the experiment (after the 12 hours of fasting), specimens II and III are centrifuged and the ratio of red cells to plasma volume in specimen II (whole blood) and the volume of red cells in specimen III (blood diluted with saline) noted. From these data it is a simple matter to calculate the total plasma volume of the rat according to the following equation: $x = \frac{ab}{c}$, where x represents the total plasma volume of the rat at the conclusion of the experiment, a is the total volume of red cells in specimens II and III, b is the plasma volume of specimen II, and c is the red cell volume of specimen II.

To determine the rat's total blood volume at the onset of the experiment, specimen I is also centrifuged, and plasma volume is calculated by the formula: $y = \frac{de}{f}$, where y equals the total plasma volume of the rat at the onset of the experiment, d represents the total red cell volume of specimens I, II, and III, e is the plasma volume of specimen I, and f is the red cell volume of specimen I. This formula will give a plasma volume y which is a

little high, inasmuch as d will be increased by red cells entering the circulation from the spleen and elsewhere during the 12 hours between the first and second bleedings of the rat.

Since total plasma protein values are obtained as the product of plasma protein concentration and total plasma volume, this high value for plasma volume is reflected in the calculations of total plasma protein, giving abnormally high values for the latter, the actual discrepancy amounting to an increase of some 10 per cent. In these experiments, protein regeneration is taken as the difference between calculated total protein at the onset of the experiment and calculated total protein at its conclusion 12 hours later. Consequently, since the method of calculation of total protein at the onset yields high values, the figures obtained for regenerated protein will be low, but will nevertheless give an indication of their true values.

The effect of splenectomy at the start of the procedure; of the intraperitoneal injection of 1 cc. of india ink at the start of the procedure; and of

TABLE 1

Average values for total plasma protein of rats before and after various procedures

PROCEDURE	NUMBER OF RATS	ORIGINAL PLASMA PROTEIN			FINAL PLASMA PROTEIN	
		Total, gm./100 sq.cm.	Withdrawn, gm./100 sq.cm.	Per cent of total	Total, gm./100 sq.cm.	Per cent of original total
Control	10	0.0981	0.0408	41	0.1021	104
Splenectomy	8	0.0941	0.0392	41	0.0921	97
India ink	8	0.0806	0.0317	39	0.0616	76
Fasting 7 days	11	0.0766	0.0336	44	0.0823	107

fasting for seven days were compared with a control series. These comparisons are valid only if the entry of red cells into the blood during the 12 hour period is roughly constant. That this is so seems probable, inasmuch as splenectomy did not change the results.

RESULTS. The results are shown in table 1. In the control series removal of 41 per cent of the calculated original protein is followed in 12 hours by regeneration to 104 per cent of the original amount. Splenectomy and fasting after removal of 41 per cent and 44 per cent, respectively, did not significantly alter this return, the regeneration being 97 per cent and 107 per cent of the original amount.

Injection of india ink, however, decreased the return to 76 per cent after removal of 39 per cent of the original amount. The peritoneal surface, lymph glands, and liver were deeply stained by the india ink.

The original hematocrits showed a consistently higher proportion of cells to plasma than the final hematocrits throughout all of the experiments.

DISCUSSION. It is seen that the return to normal of the total blood protein in a short period after a severe bleeding far outstrips the return of red cells. The site of origin for this remarkable influx of protein is uncertain. However, since the injection of india ink decreases it, it may be supposed either that the reticuloendothelial system is responsible for its prompt manufacture, or, less likely, that the india ink has blocked lymphatic channels and prevented entrance of tissue-space protein into the circulation.

CONCLUSIONS

1. Twelve hours after removal of 40 per cent of the blood, total blood proteins return to the original amount.
2. Injection of india ink decreases this protein regeneration.

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CHANGES IN THE CIRCULATORY EFFECT OF POTASSIUM SALTS DUE TO EPINEPHRINE (ADRENALIN)

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In 1911, Mathison (1) found that intravenous injection of potassium salts causes a markedly different effect than the intra-arterial injection. Intravenous injection causes a primary fall of blood pressure followed, if the dose be not too large, by a relatively small secondary rise. Mathison found also, that potassium salts injected intra-arterially, elicit a pronounced rise of blood pressure which resembles the response to epinephrine. We have confirmed these findings.

The point we wish to add to Mathison's work is, that the intravenous injection of a potassium salt immediately after, or within a short time after adrenalin, will elicit almost as great a rise in blood pressure as will the intra-arterial injection. In studying this response we have found also that KCl alone intravenously under certain conditions will give a great rise in pressure. The action after adrenalin seems to be due to an increase in the potassium content of the blood.

There seems to be a direct relationship between adrenalin activity and the potassium of the tissues. Heinrich Schwartz (2) claims that 0.25 mgm. per kilo of adrenalin intravenously in rabbits causes an increase in blood K as much as 86 per cent—which is inhibited by curare and ergotamine. The part played by KCl in the blood pressure rise is not discussed.

EXPERIMENTS. Dogs were anesthetized with pentobarbital about 35 mgm. per kilo body weight. Injections were made into the femoral vein, and in case of arterial injections, the femoral or carotid artery was used, but the best results were obtained by injecting into the arch of the aorta.

That this effect is due directly to the KCl and that a definite threshold of KCl is necessary to produce the action is indicated in the following experiments.

Group I experiments. In this group of experiments the following facts were determined:

1. Sensitizing doses of cocaine do not increase the response hence the effect is not due to liberation of sympathin E.

2. Repeated doses of KCl do not increase or decrease the epinephrine content of the adrenal glands as determined by biological assay.

Group II experiments. Keith and Binger (3) have shown that K salts are readily excreted in the urine, and Biernacki (4) states Na salts hasten the excretion of K salts. Amberg and Helmholz (5) have shown that NaCl detoxifies to some extent the effect of KCl. The appended experiment shows the combined effect of flushing out the KCl and the detoxifying action of Na.

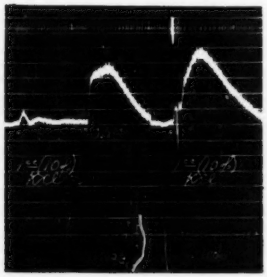

Fig. 1. Shows the blood pressure raising action of KCl before and after 0.5 cc. 1:50,000 adrenalin hydrochloride solution.

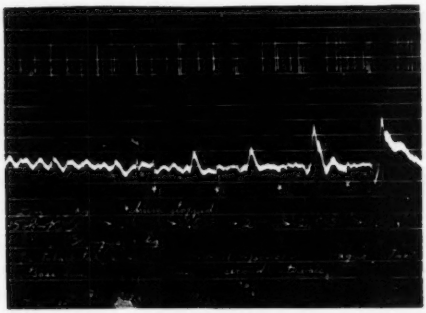

Fig. 2. Shows the progressive action of KCl given intravenously without any previous medication.

July 3, 1935. In a dog weighing 11 kilos we injected intravenously within 30 minutes, 275 cc. 8 per cent Na_2SO_4.

The volume of urine excreted in two hours was 635 cc. After this, the reaction to adrenalin was markedly reduced.

Repeated doses of adrenalin were given, after which KCl intravenously did not cause a rise of blood pressure, as it does ordinarily. An injection of 5 cc. 5 per cent KCl into the aorta did cause a rise but not so great as normal. A second injection into the vein caused some rise.

A second injection into the aorta caused a much greater rise than the first. After this, injections into the vein also caused a rise. The amount

of KCl to restore something near a normal response to adrenalin was 15 cc. of 5 per cent solution. Our explanation is that the Na_2SO_4 had reduced

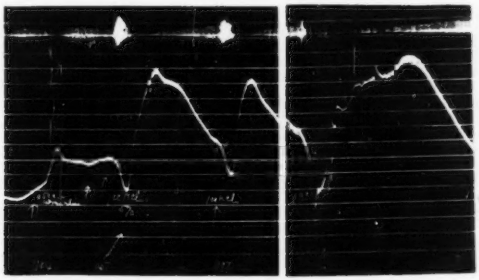

Fig. 3. Shows the action of 1 cc. of KCl given intravenously, one minute after 0.5 cc. 1:50,000 adrenalin hydrochloride solution, and 24 minutes after there having been 12 intervening 1 cc. doses of KCl.

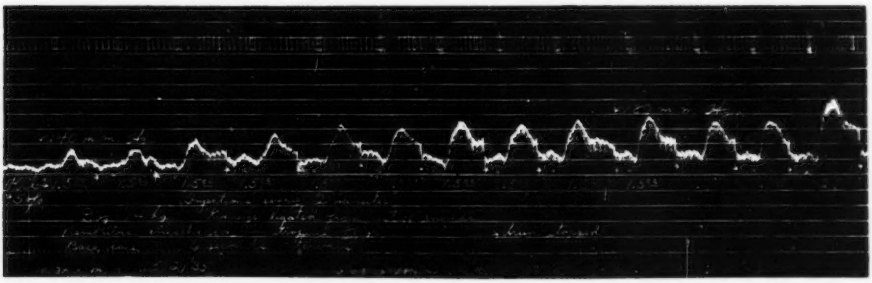

Fig. 4. Shows the effect of gradually increasing the concentration of KCl in the blood intravenously.

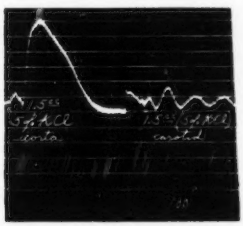

Fig. 5. Shows comparative action of 1.5 cc. 5 per cent KCl when injected into the aorta and into the carotid artery.

the KCl in the body to a level that was below the threshold necessary for the adrenalin-like action. Massive doses of NaCl intravenously will also

eliminate the response to adrenalin which to some extent at least may be returned by potassium.

Group III experiments. Potassium salts cause a constriction of the vessels and smooth muscles (Mathison), and a depression of the heart. When injected intra-arterially, especially into the aorta, they act mainly on the arterioles and reach the heart so dilute that no great effect is produced. When injection is made into the femoral vein, they reach the heart in a concentration that weakens the heart to such a degree that the blood pressure is lessened. In addition, potassium reaches the peripheral arterioles in such a dilution that the vaso-constrictor effect is less. Large doses of KCl intravenously, however, if they do not paralyse the heart cause a marked rise of pressure (see fig. 1).

We have, therefore, tested the effect of raising the concentration of KCl in the blood slowly so that it would not immediately paralyse the heart. This was done by injecting intravenously a dose of 1 or 1.5 cc. of a 5 per cent solution in a 10 kilo dog every two minutes. The results were that after a few injections we obtained a response corresponding to the effect when the salt was injected into the artery (fig. 2). To raise the concentration of the KCl in the blood stream, it is necessary to inject repeatedly and the injections must be within a few minutes of each other (fig. 3) because of the rapid disappearance of the salt from the circulation. This also explains why we found that the result was obtained in about two minutes after the injection of adrenalin. To get a blood pressure rise from potassium chloride, therefore, all that is necessary is to raise the concentration of it, in the blood, and at the same time keep that concentration below the concentration that will paralyse the heart. Figure 4 shows this is quickly obtained when the renal arteries are ligated.

Site of action. That the action is peripheral and direct on the blood vessel muscle is indicated by the following:

Decapitation. A dog was decapitated, after the vessels to the head had been ligated, so that there was little bleeding. After adrenalin, KCl caused a distinct rise, but not so great as in the normal animal. The blood pressure was low and the response to adrenalin was also less than in the intact animal. The action occurs after atropine, nicotine and after removal of the adrenals. Mathison found it also occurred after ergotoxine. The main action is, therefore, peripheral, and apparently on the arterioles.

The minimal effect produced by injection of KCl into carotid artery as compared with the marked reaction from injection into aorta suggests that the action is not due to a carotid sinus reflex (fig. 5).

Therefore since the effect is not exaggerated by sensitizing doses of cocaine, and since it occurs after atropine, curare, and is not of carotid sinus origin it is concluded that the site of action is peripheral, directly on the wall of the arterioles and capillaries.

CONCLUSIONS

1. The work of Mathison on the action of K salts is confirmed.

2. The rise in blood pressure effect by K salts alone or after epinephrine is due to a direct action on the blood vessel wall.

3. An increase in K content of the blood is necessary before the blood pressure raising action is effected.

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STUDIES ON THE EXTRINSIC AND INTRINSIC NERVE MECHANISMS OF THE HEART[1]

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In a previous paper the initial inotropic and the chronotropic effects produced on the sinus and atrium of the turtle heart by vagus nerve stimulation were associated with different groups of fibers. It was inferred on the basis of the evidence there presented that the fibers responsible for the initial inotropic effects were thinly myelinated, those responsible for the chronotropic effects unmyelinated. Herein are recorded results of further investigations of this problem in the turtle and in the cat. Evidence is presented consistent with the interpretation that in the turtle the inotropic and chronotropic mechanisms are separable, the latter not representing simply a further degree of depression of the former, but that this separation does not correspond sharply with the myelinated-nonmyelinated division. Certain pharmacological studies on the turtle and Limulus hearts using nicotine sulphate as a depressant are reported.

It was found by us (Heinbecker, 1931) and by Lee (1935) that in a small percentage of turtle preparations using Pseudemys elegans, P. concinna and P. scripta, it was not possible to separate by thresholds an inotropic from a chronotropic effect on the sinus and atrium by stimulation of the right cervical vagus trunk. When using as stimuli shocks of short duration the number of such preparations is fewer than with shocks of longer duration, a finding which depends upon differences in threshold separability of the various nerve fiber groups with different forms of stimulating current. In this and in previous investigations discharges of condensers ranging from 0.01 to 0.1 microfarad were employed. In a few such preparations when the potential record was observed coincidentally with the myographic record it was found that both inotropic and chronotropic effects were being obtained when only the B_3 potential maximum was being elicited. In a still smaller percentage of preparations neither an inotropic or a chronotropic effect was obtained until the first portion of the C potential appeared. *In the vast majority of turtles of all species studied* the initial inotropic effect

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was associated with the B_3 potential, the entire chronotropic effect with the C potential. No evidence as to the relative efficacy of single fibers from the B_3 and C fiber groups is available here, but elsewhere it has been shown that larger fibers of a similar group may have a greater physiological effect than small fibers (Bishop, Heinbecker and O'Leary, 1934). If the inotropic

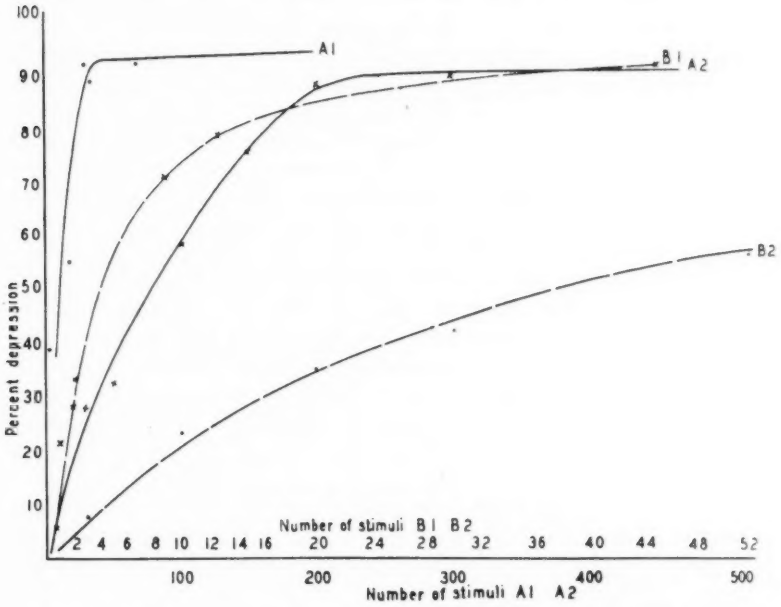

Fig. 1. *A1, A2.* Inotropic depression of turtle heart from stimulation of the right vagus nerve; plots of percentage depression against number of stimuli. *A1.* Stronger stimulus, 19.5 volts, just above threshold for chronotropic effect. Stimulation rate, 1.8 per second. *A2.* Weaker stimulus, 16.5 volts, rate 5.5 per second, just below threshold for chronotropic effect. In *A1* slowing took place after 85 stimuli, in *A2* no slowing at any duration, even at 3 times the frequency. By stimulating purely inotropic fibers in *A2* more rapidly the same degree of inotropic depression is obtained as by stimulating more fibers, some of which are chronotropic, more slowly. *B2, B2.* Same, but rate constant at 3.4 per second. In *B1*, slowing after 60 stimuli at 41.5 volts. In *B2*, no slowing at any duration at 38.5 volts.

and chronotropic mechanisms are separable, not merely different degrees of the same effect, then in those occasional preparations where low threshold fibers have only an inotropic effect at any rate of stimulation it should be possible to test this difference. That is, if the *same degree* of inotropic depression were to be produced by each group of fibers the one group might also produce a chronotropic effect, the other not. This is found to be the

case. With slow stimulation, 1 per second, the degree of inotropic depression obtainable from B_3 stimulation without any chronotropic effect varied from 0 to 60 per cent of the maximum obtainable from stimulation of all fibers at that rate. With faster stimulation, 4 to 8 times per second, in certain preparations up to 90 per cent or more of the possible maximum inotropic depression at that rate could be obtained without any chronotropic depression (fig. 1). With the faster rates of stimulation of those fibers (B_3 in certain preparations) from which no chronotropic effect is obtained regardless of the rate and duration of stimulation it was shown that the form of the curve obtained on plotting the percentage of inotropic depression against the number of stimuli is similar in form and amplitude to the curve similarly obtained on stimulating at a slower speed all the B_3 and some of the C fibers, thereby producing both an inotropic and a chronotropic effect. The stimulus strength and frequency employed were such as to result in a moderate chronotropic depression after 15 to 30 seconds of stimulation. By temporal summation in one fiber group an inotropic effect was thus obtained identical with that resulting from the additional spatial summation when two fiber groups were involved. Because of the degree of inotropic depression obtainable, 90 per cent or more in certain cases, it is evident that inotropic depression is not simply a lessened degree of depression of some mechanism, the further depression of which necessarily results in a chronotropic change. The above findings apply to the experiments involving the right vagus nerve.

On the left side the findings were in general similar to those on the right as far as inotropic effects were concerned. But not infrequently slight, if any, chronotropic effect was obtained on stimulation of even a maximal C wave. Apparently the chronotropic fibers of each nerve are distributed principally to the pacemaker mechanism of the ipsolateral side. The right side being the usual locus of the controlling pacemaker, it follows that the right nerve is chronotropically more effective than is the left. When the pacemaker mechanism becomes left sided, then the left nerve is equally effective as a chronotropic nerve (Garrey, 1911).

The action of nicotine on the vagus mechanism of the turtle and Limulus polyphemus. To test further this separability of the inotropic and chronotropic mechanisms experiments were conducted in which nicotine (1 cc. 1 per cent in 50 cc. Ringer's solution) was evenly applied to the turtle sinus and atrium by immersion. The preparation was so arranged as to permit stimulation of the right cervical vagus and the myographic recording of the sinus and atrial contractions. It was found that the chronotropic effect of nerve stimulation was lost in the sinus and atrium before any appreciable inotropic depressibility was lost. However, both effects disappeared in a few minutes, before any appreciable depression of the heart musculature occurred as evidenced both by its contraction height and its

irritability to electrical stimuli. The nicotine solution was then washed off and Ringer's solution repeatedly applied to the preparation. On recovery it was generally possible to obtain by right vagus stimulation a chronotropic depression of both the sinus and atrium without any inotropic depression; in fact, due to the slowing, the force of the sinus and atrial beats became stronger (fig. 2).

The statement that nervous tissue is more susceptible to the depressing action of nicotine than is muscle tissue is generally accepted. The brief interval, seconds rather than minutes, required to eliminate all extrinsic vagus nerve effects by $\frac{1}{2}$ per cent nicotine at a time when the heart beat itself is little affected, seems to indicate that the extrinsic vagus nerve fibers act through an intrinsic nerve mechanism rather than on musculature directly. However, the possibility remains that the point of action may be at the so-called myoneural junction. The relative susceptibility to nicotine depression of the myoneural junction as compared with other structures was therefore investigated.

To obtain such evidence studies were carried out on the heart of Limulus polyphemus. When nicotine is applied to the excised median cardiac nerve ($\frac{1}{2}$ per cent neutralized nicotine sulphate in sea water) the effect is to quicken the frequency of the autochthonous ganglionic discharges. Occasionally a transitory slowing for a few seconds is encountered. Along with the quickening there is some depression of the amplitude and duration of the potentials associated with each rhythmic discharge. Later the frequency of the rhythmic discharge is gradually lessened until finally in 15 to 30 minutes all discernible activity in the ganglion cells disappears (fig. 3). When the whole excised Limulus heart is placed in such a solution of nicotine its myographic record shows changes corresponding to those observed in the excised median cardiac nerve (fig. 4). If the preparation is so arranged that the electroneurogram recorded from the elevated caudal portion of the median nerve cord is observed on the face of the oscillograph coincidentally with a heart myogram it is seen that the changes in activity of the whole heart are parallel to changes in activity of the ganglion cells. If $\frac{1}{2}$ per cent nicotine is applied to the median nerve cord the effect of the extrinsic fibers, inhibitory and excitatory, are eliminated in a few seconds. For minutes (5 to 15 or more) the heart can still be driven by stimulating the median nerve cord directly. After cessation of spontaneous beats (5–15 minutes or more) the heart can still be excited to a generalized contraction by direct electrical stimulation.

Certain conclusions may be drawn from these observations. 1. The synapses between the extrinsic nerve fibers and the spontaneously rhythmic ganglion cells of Limulus are similar in their susceptibility to nicotine block to the synapses of the sympathetic ganglia of such animals as the turtle and cat, and are similar also to the corresponding mechanisms of the turtle

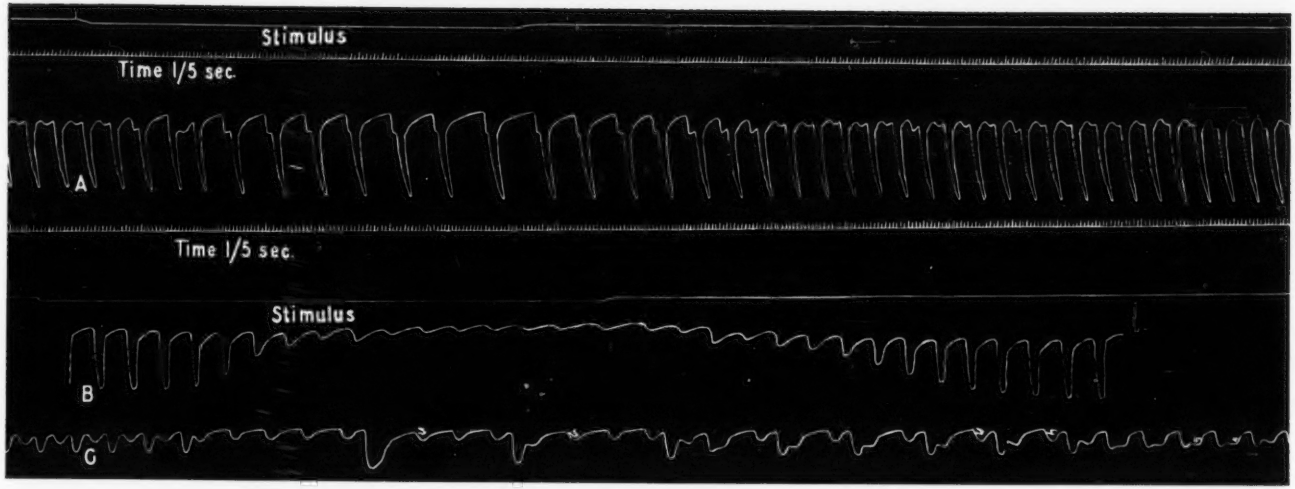

Fig. 2. A. Myogram turtle sinus and atrium showing chronotropic depression from stimulation of the right vagus nerve during recovery from nicotine depression, one-half per cent. Note absence of inotropic depression of either sinus or atrium. With the slowing there is actually an increase in the strength of the beat of both sinus and atrium.

B. Record showing normal recovery of sinus and atrium from depression caused by right vagus stimulation.

C. Myogram of turtle sinus and atrium showing the effect of right vagus stimulation during recovery from nicotine poisoning. Note a blocking of conduction between sinus and atrium with no apparent inotropic depression of the sinus. The atrial beats, when they occur, are stronger than before stimulation indicating no inotropic depression. This record is to be contrasted with record B showing the normal form of recovery from vagus stimulation where both inotropic and chronotropic depression have been present.

heart. 2. The rhythmical ganglion cells are depressed by nicotine much more slowly than are their extrinsic synapses, the time required for complete depression approximating that required for nerve fibers. 3. The internal coördination of the ganglion cell mechanism of Limulus is not deranged by nicotine as would be anticipated if synapses of the type known elsewhere in the peripheral sympathetic nervous system were in-

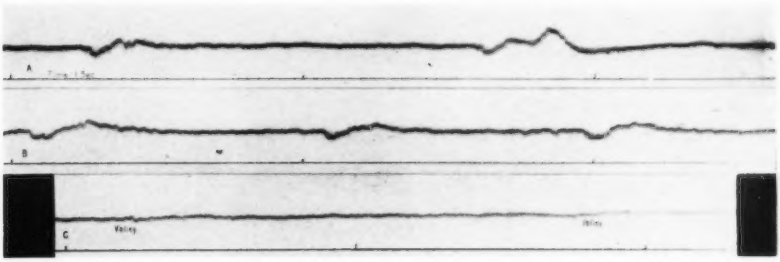

Fig. 3. Electroneurogram of excised median cardiac nerve of Limulus polyphemus after the application of one-half per cent neutralized nicotine sulphate to the entire nerve cord. Figure contains three portions of a continuous record. *A*. Normal. *B*. Three minutes after nicotine applied. *C*. Eighteen minutes after nicotine applied. Note first in *B* an increase in frequency of the volley discharges with a lessening of their total duration followed in *C* by a slowing of the rate of volley discharges together with a marked depression in amplitude and duration of the potential complexes.

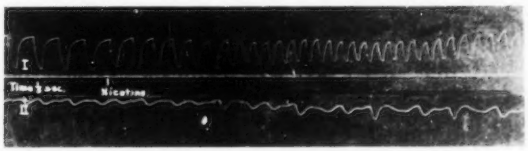

Fig. 4. Myogram Limulus heart showing the effect of one-half per cent neutralized nicotine sulphate applied to the median cardiac nerve. Note first the increase in rate with some inotropic depression later followed by a decrease in rate and further inotropic depression. The two parts of the record are not continuous, an interval of approximately fifteen minutes has elapsed between I and II. The effect is similar on the exised heart.

volved. Apparently the nerve network functions more like that in the central nervous system of mammals. 4. The so-called myoneural junction is not as susceptible to nicotine block as is a sympathetic synapse. It is apparently no more susceptible to nicotine depression than is the nerve fiber of comparable size itself.

When nicotine is applied to the turtle sinus and atrium in concentrations

varying from 0.5 to 0.005 per cent the effect is to produce a lowering of the contraction height and a quickening of the rate. In a few preparations there is a transitory slowing with possible irregularity of rhythm. The increase in rate is followed by a gradual slowing with an increase in contraction height. As the depression increases the heart slows and the force of the beat is lessened until finally spontaneous activity ceases. At this stage of depression it is still possible to obtain a well developed and conducted contraction of sinus and atrium by direct electrical stimulation of the sinus. The time required to stop spontaneous activity in an excised turtle heart varies greatly (15 to 30 minutes or more). This approximates the time required to depress to extinction conduction in a nerve fiber of the turtle or in the Limulus median cardiac nerve. The time for depression to extinction of autochthonous ganglion cells is also of this order. The time required to block the effect of extrinsic nerve fibers on the intrinsic inotropic and chronotropic mechanisms is roughly 1/30 to 1/60 of that required to stop spontaneous activity of the autochthonous ganglion cells of Limulus. This is also the ratio of the times consumed in blocking the synapse and nerve fibers of the sympathetic system of the turtle. The time necessary to depress muscle so that it will no longer contract and conduct is appreciably longer by 50 to 100 per cent or more. Carlson (1909) has reported similar findings with regard to the relative depressibility of ganglion cells, nerve fibers and muscle with nicotine in the invertebrates.

By all the criteria then which the action of nicotine gives evidence of, the mechanism not only of the spontaneous beat, but of the effect on it of the extrinsic nerves, is similar in turtle and Limulus, and in Limulus the destructive action of nicotine is demonstrably on nervous structure and not on muscle. The obvious conclusion is that if the turtle heart beat is not essentially neurogenic, then the pacemaker muscle in acquiring its characteristic properties has acquired properties that are the attributes elsewhere of nervous tissue.

Fibers responsible for vagus inhibition in the cat's heart. The potential maxima associated with the chronotropic effect in the mammal vagus were also studied in the rabbit and cat. Under ether anesthesia a tracheal cannula was inserted and both cervical vagus trunks were completely dissected out in the neck. They were then cut just below the nodose ganglion. A small chamber containing both stimulating and recording electrodes enclosed the right or left vagus trunk as desired. Changes in heart rate were read from a blood pressure record obtained with a mercury manometer on cannulating the femoral artery. By observing the neurogram it was possible to observe threshold and maximal responses associated with the various potential components and thus correlate potential form with functional effect on the heart rate.

Typical results are plotted in figures 5 and 6. It was found that excita-

tion of the B_1 potential was without effect on the heart rate or blood pressure. The chronotropic effect on the heart was initiated on excitation of the fibers giving rise to the B_2 potential which is the autonomic motor component of the myelinated fiber content of the vagus; and only the slowest half of this potential is connected with the heart effect. An almost maximal effect was obtainable from this potential on both the right and the left sides. Ten per cent or less increase of chronotropic effect was frequently obtained on adding the C potential to the B potential. In certain preparations very little additional effect was obtained from the C potential.

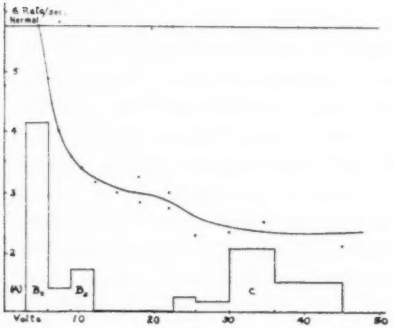

Fig. 5

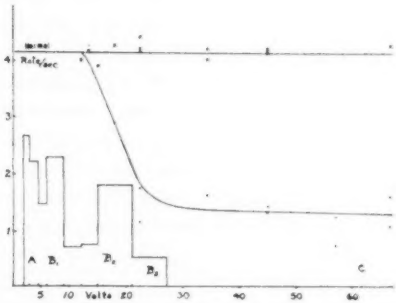

Fig. 6

Fig. 5. Plot of potential area against stimulus strength right vagus nerve of rabbit and the heart rate at the various stages of development of the potential area. Note that slowing begins only after fully half of the B_2 potential has been developed. Most of the chronotropic effect is the result of activity in the myelinated fibers giving rise to the B_2 potential. In this preparation some further slowing results from activity in the fibers giving rise to the C potential. Rate of stimulation 14 per second.

Fig. 6. Plot of potential area against stimulus strength right vagus nerve of cat and the heart rate at various stages of development of the potential area. Note that slowing does not begin until the B_2 potential is developed. In most preparations slowing does not begin until half of the B_2 potential is developed. In this preparation there is no further slowing from activity in the fibers giving rise to the C potential which is not plotted, but its position indicated by *C*. Rate of stimulation 32 per second.

The interpretation of our results depends upon the accuracy and validity of the correlations between fiber groups and action potentials previously described (Bishop and Heinbecker, 1930). Evidence has been presented that there are two main axon types, the one with fast properties characteristic of the somatic nerve fibers, and the other with slow properties characteristic of the autonomic nerve fibers (Heinbecker, 1929). The somatic nerve fibers give rise to the A and B_1 potentials. These fibers are

all myelinated and their size range extends from the largest fibers in the nerve trunk down to those having a diameter of 2 or 3 microns. The myelinated autonomic fibers range from 4.5 to 1.5 microns approximately and give rise both to the B_2 and the B_3 potential maxima according to the evidence now available (Douglas, Davenport, Heinbecker and Bishop, 1934). These investigators have plotted fiber sizes in the turtle vagus for comparison with action potential waves. The B_3 potential maximum definitely appears to be derived from the myelinated fibers, 3 to 1.5 microns in diameter, the C potential from non-myelinated fibers. Similar correlations appear to hold in the mammalian vagus nerve. In the turtle the conduction rate of the fastest fibers of the B_3 potential ranges from 1.7 m.p.s. to 1.2 m.p.s., of the C potential from 0.6 m.p.s. to 0.4 m.p.s. Their absolutely refractory periods correspond ranging from 3.5 to 5 sigmas. In general the absolutely refractory period values for the myelinated autonomic fibers tend to approximate the lesser of these values, those for the unmyelinated fibers the greater. In the cat the average conduction rate of the slowest myelinated fibers is not over 2.5 meters per second.

A correlation of our experimental findings with the above data, then, indicates that in the turtle myelinated autonomic fibers (B_3) are responsible for the initial inotropic effect which is obtainable without an associated chronotropic effect in most preparations, but non-myelinated fibers have also a definite inotropic effect. In certain nerves the number of myelinated inotropic fibers is limited so that it becomes more difficult to separate out an appreciable inotropic effect by electrical stimulation because on increasing the strength of stimulus non-myelinated fibers are soon activated and this results in both inotropic and chronotropic effects being added simultaneously. In the preparation where no effect on the heart is recognized until a C potential is visible it is inferred that only non-myelinated fibers were distributed to the heart. In those occasional preparations where both inotropic and chronotropic effects are obtained from the B_3 potential, it is to be inferred myelinated fibers serve both inotropic and chronotropic effects. In the cat myelinated fibers are chiefly responsible for both inotropic and chronotropic effects. Non-myelinated fibers are to a slight degree also chronotropic.

It is stated by Brown and Eccles (1934) that the vagus fibers in the cat which affect the heart have a conduction rate up to 30 meters per second which would put them in a range that we have found to be occupied by somatic fibers only. The fastest of the autonomic motor fibers of the cat vagus conduct at not over 15 meters per second and those usually affecting the heart at not over half of that. The differences we find between turtle and cat are not that different groups of fibers in these animals produce a given effect, but rather that of the two groups of fibers, myelinated and non-myelinated autonomic, the latter is involved predominantly in the

turtle, especially with respect to the chronotropic action and the former in the cat. Even in the turtle it is evident that there is no sharp division between these groups of fibers as judged by their effects on the heart and there is even less basis for a functional division in the cat. As we have pointed out before, the main functional division lies between autonomic and somatic fibers, not between myelinated and non-myelinated.

Discussion. The interpretation of the experimental finding that a slowing of the sinus and atrium of the turtle heart can result from vagus stimulation during recovery from nicotine, before the inotropic susceptibility has recovered, involves the much disputed question of the nature of the pacemaker mechanism itself. If the pacemaker is muscular, then it might be expected that depression of force of beat by the vagus would only affect rate if the fibers involved were distributed to the sinus itself, and the difference of effect would only be a difference in the locality affected.

An ultimate muscular pacemaker is, however, so difficult to discover histologically, to say nothing of recording from it, that Brown and Eccles (1934) have been forced to relegate it to a limbo where it could not be detected even if it were a fact, that is, entwined in the interstices of the other and less authochthonous heart muscle fibers. On the other hand, it is perhaps significant that in the turtle atrium those parts that originate the rhythmic beat when the sinus is destroyed are inhabited by ganglion cells, and that in the ventricle proper, which in the turtle contains no nerve cells, a spontaneous beat does not originate after the funnel tissue containing ganglion cells is removed.

The recent statement of Eccles and Hoff (1934) to the effect that there is no longer any basis for a belief in a neurogenic mechanism of the heart beat is therefore not convincing. The possibility that the depression of a restricted region of specialized muscle tissue (pacemaker) might not be apparent in a myographic record of the sinus and atrial contractions is recognized. But if the slowing of the pacemaker mechanism without apparent inotropic depression is a possibility then it becomes highly improbable that the pacemaker is a muscular structure. With rhythmical muscle tissue there should be first an inotropic depression which would progress into a chronotropic one. In Limulus it has been shown by Hoffman (1911) and Heinbecker (1933) that such an order of depression holds even for ganglion cells with an autochthonous rhythm. First the duration of the volley discharge is lessened and then on further depression the frequency of volley discharges is reduced. If the turtle possesses a ganglionic pacemaker it must of necessity differ somewhat from that of Limulus in that the type of response to its activity has changed to one of an all or none character from one of a tetanic character. This makes unnecessary a repetitive form of ganglionic discharge. Single discharges from rhythmical ganglion cells would serve. In keeping with this are the results of experiments in which

stimuli up to 200 per second were applied to the turtle sinus so timed as to drive it and continued throughout the period of contraction. This failed to alter the form of the contraction from that resulting from single stimuli. The rhythm of intrinsic ganglion cells could have been altered without any effect on the myographic record.

In Limulus it has been shown that extrinsic nerve impulses alter the rhythm of the autochthonus cells without imposing their rhythm on them. Phylogenetically such an arrangement would be expected to hold also in the turtle heart if it contains rhythmical ganglion cells.

Inotropic modification also gives evidence of a nerve mechanism because of the rapidity with which its connections with the extrinsic nerve fibers are blocked by nicotine. Supporting evidence for such an inference has been furnished recently by Armstrong (1935) who has demonstrated that in developing Fundulus hearts sensitization to acetylcholine does not occur until its innervation is completed. No definite knowledge of the inotropic mechanism in the turtle is available. In Limulus it was found, if chronaxie be taken as a criterion, that the musculature is not inotropically altered by removal of its ganglionic connections, a result in disagreement with the findings of Dubisson (1931). Any inotropic effect is therefore presumably transmitted to the musculature by the excitation of ganglion cells by extrinsic nerves in which there would presumably be a one to one relationship between the pre- and postganglionic connections as in ordinary sympathetic ganglia likewise without spontaneous activity. The evidence presented indicates that the inotropic mechanism can be altered separately from the chronotropic mechanisms by the extrinsic nerves.

SUMMARY

Evidence is presented to the effect that in the turtle the extrinsic vagus fibers responsible for the negative inotropic effect are both myelinated and non-myelinated. The fibers for chronotropic effects are mostly non-myelinated, a few are myelinated. All fibers having inotropic and chronotropic effects have axons with slow properties characteristic of autonomic fibers in general.

In the cat the extrinsic vagus chronotropic fibers are mostly myelinated, a few are non-myelinated. The inotropic fibers are inferred to be myelinated essentially. Both the myelinated and non-myelinated fibers concerned have properties typical of autonomic fibers in general.

For reasons discussed, inotropic and chronotropic fibers are considered to be functionally distinct.

Nicotine sulphate ($\frac{1}{2}$ to 2 per cent in Ringer's solution) applied to the excised median cardiac nerve of Limulus polyphemus causes first an increase in the frequency of the rhythmic volley discharges and then some shortening and lowering of the potential complex. Further depression

results in a decrease in frequency of the volley discharges, further shortening and lowering of the potential complex. The heart of Limulus is stopped by nicotine when the activity of its nerve cells ceases. This requires 5 to 15 or more minutes when ½ per cent nicotine is used.

The blocking effect of nicotine on the synapses intervening between the extrinsic and intrinsic nerves of the Limulus heart is similar to its blocking effect on other synapses of the peripheral sympathetic nervous system of vertebrates. One-half per cent nicotine blocks them in 15 to 30 seconds.

There is no evidence to indicate that the intrinsic nerve mechanism of Limulus polyphemus contains synapses of the type associated with the extrinsic to intrinsic nerve relations. The intrinsic nerve mechanism of Limulus polyphemus functions in this respect like the central nervous system of vertebrates.

The myoneural junction is not blocked by nicotine in the period of time required to block an ordinary nerve synapse. It is similar to the time required to block a nerve axon of comparable size. Heart musculature is depressed still more slowly than are nerve elements.

The effect of nicotine on the turtle heart seems comparable to its effect on the heart of Limulus.

By analogy from evidence derived from Limulus studies the extrinsic nerves of the turtle are considered to act through an intrinsic nerve mechanism and not directly on muscle.

On recovery from nicotine depression it is possible to obtain by right vagus stimulation a slowing of the sinus and atrium of the turtle with no evident inotropic depression of either structure.

The significance of the findings in these turtle and Limulus studies in their relation to the problem of determining the nature of the pacemaker mechanism of the heart is discussed.

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THE ELECTRICAL PHENOMENA OF THE CRUSTACEAN NERVE-MUSCLE SYSTEM

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I. The nerve-muscle system in crustacean claws consists of antagonistic muscles with complex innervation. At least four types of theory have been advanced to describe the properties of these antagonistic systems.

The best known of these was proposed by Biedermann (1888) who observed that the adductor (closing muscle) and abductor (opening muscle) of the propodite moving the dactylopodite have a double innervation from nervus medius and nervus externus pedis. The nervus medius (thick nerve trunk) innervates mainly the adductor; he believed that stimulation of this nerve causes contraction of the closing muscle. Some of its fibers join the nervus externus and, according to his view, cause inhibition of the opening muscle. The nervus externus (thin nerve trunk) innervates the opening muscle in a similar way.

Richet (1882) attributed the differences to a difference in properties of the nerve-muscle systems. He was more or less supported by Mangold (1905), Lucas (1907) and Monnier and Dubuisson (1931).

Another group denied the existence of inhibitory nerves, e.g., Froehlich (1907), Fraenkel-Conrat (1933). Segaar (1929) concluded that each nerve can excite or inhibit depending on the strength of stimulation.

According to a fourth group the interaction of peripheral nerve cells is an explanation for the complex phenomena in the crustacean claw (von Uexkull and Tirala, 1915; Tonner, 1933). Barnes (1932) explained the phenomena by the fact that the amputation of the claw causes tetanotonus and other forms of anomalous behavior.

The following paper is an attempt to reconcile the several theories and to consider excitation and inhibition from a single point of view based on an analysis of the properties of these muscles and nerves.

II. *Methods.* The electrical responses of nerves and muscles of the claws of several Crustacea were observed by means of a string galvanometer and amplifier (Garceau and Forbes, 1934), a cathode-ray oscillograph with amplifier (Garceau and Davis, 1934), or two undulators with amplifiers (Garceau and Davis, in press) and preamplifiers (Matthews, 1934).

Recording electrodes were: agar electrodes, chlorided silver wires, or

concentric needles (steel-copper, silver-silver, etc.). In some experiments a fine silver wire with a blunt end, bent at an angle of 90°, and then coated with varnish, was used. After baking, the varnish was scraped off over a minute area on the inner side and this area was chlorided. When such an electrode is put under or in a nerve or muscle group while a diffuse electrode serves as ground, only the action potentials of the nerve or muscle fiber in close proximity to the minute area are detected (fig. 9). This type of electrode does not injure the tissues, as often happens with concentric needles. The mechanical responses were recorded on a long paper kymograph with an isotonic lever.

A Harvard coil provided electric stimulation; reflex stimulation was given by tapping on the outside of the claw, on the antennae, or on the tail to induce opening, and by touching the inner side of the claw to induce closing.

Solutions of several drugs were injected through holes in claws either isolated or in the intact animal. The general behavior of the animals was observed while they were in a quiet or circulating solution.

In several specimens the drug was injected into the tail. Records were taken from intact animals or from isolated claws kept in a thermostat at high humidity at 5 to 10°C. for lobsters, and at 15 to 20°C. for crayfish.

It might be objected that the electrodes would pick up disturbances from a distance. Actually this is not the case for: *a*, the two muscles are separated by a membrane; *b*, records of the antagonistic muscles were made simultaneously with the two independent undulators and different patterns were found in the two muscles (fig. 1); *c*, a concentric or a coated needle gives essentially similar responses from similar positions; *d*, when an electrode is placed in a region that is mechanically damaged, no action potentials can be picked up.

III. *The nature of crustacean muscle.* The muscles of the crustacean claw belong histologically to the striated muscles. Jasper and Pezard (1934), however, state that for Carcinus maenas the size of striation varies with the muscle: the faster the muscle, the shorter the distance between striations. On the other hand they show some characteristics of smooth muscle. In the first place, they are doubly innervated by motor axons. [According to Tonner (1933) there is in addition a third fine innervation from peripheral nerve cells.] Secondly, normal vertebrate striated muscles do not react to adrenalin or acetylcholin, whereas crustacean muscles contract when treated with adrenalin, although not with acetylcholin. The following typical experiments show this.

March 8, 1935. Isolated claw of Cambarus. All nerves cut in procarpopodite joint; 0.1 cc. sol. adrenalin chloride (P.D. & Co. 1:1000) in 0.9 cc. crustacean Ringer (double mammalian Ringer) injected between adductor and abductor through a hole in the propodite. Contraction without action potentials was observed.

February 21, 1935. Isolated claw of Cambarus. 3:00 p.m. Stimulation by induction break shocks. Threshold for opening claw at 12 cm. coil distance; for closing, 10 cm. Crystal of acetylcholin put in through hole in propodite. No contraction follows. Reactions to break shocks remain the same for several minutes with no change of threshold. Claw dead at 5:00 p.m.

The contraction induced by adrenalin is not accompanied by any electrical change. Rosenblueth, Lambert and Leese (1933) show the same for mammalian smooth muscle. Differences in behavior can be explained by the hypothesis that drugs act at a later stage of the excitation of muscle than does the nerve impulse (Cannon and Rosenblueth, 1933), and that different muscles are activated by different drugs. The same variability in activation is true for electric stimulation (Forbes, 1933).

Furthermore the action potentials following electric stimulation of the nerve show the characteristics of smooth muscle, as is shown in figure 2.

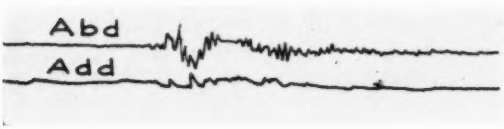

Fig. 1

Fig. 2

Fig. 1. Cambarus, intact animal. A pair of recording chlorided Ag-electrodes in adductor and in abductor. Recording with two independent undulators. Activity after reflex stimulation.

Fig. 2. Cambarus. Thin nerve cut between carpo- and meropodite. Recording gelatin electrodes in adductor, ground electrode distal. String galvanometer with amplifier, capacity coupled. Sensitivity 100 mm./mv. Stimulating electrodes in ischiopodite. The first small spike is an artefact due to the break shock.

This is a typical galvanometer record which clearly shows two components. These were observed to be the same in many records made with the oscillograph as well as the galvanometer, both for the closing and the opening muscle (e.g., fig. 3 and fig. 7; the small components are indicated by arrows). The reason why the two components were not previously observed may be because in many cases the first spike is not very distinct (being small and very rapid) and therefore may be easily overlooked.

It is improbable that the first spike is due to the action potential either of the nerve or the end plate because of the following:

1. In recording from vertebrate striated muscle with intact nerves the first spike is never recorded.

2. The time relations of the two waves always remain the same.

3. As can be seen from figure 8 the record of the true nerve action potentials shows a great difference in time relations and size as compared with the first spike.

4. The action potentials of vertebrate smooth muscle show exactly the same shape.

It is more reasonable, therefore to explain the two waves according to the theory of chemical mediation. This means that the immediate response of the muscle to nerve excitation corresponds to the small spike and that the process of liberation of the chemical substance is related to the slow wave (Cannon and Rosenblueth, 1933). Transfusion experiments, however, have not yet given positive results.

I have tried also to study the regeneration of the nerves, following section. Generally the operated limb was autotomized, but in three cases the limb was not thrown off. After about fourteen days the muscles were degenerated to structureless jelly-like masses.

IV. *Electric phenomena of the muscles.* Electric responses were measured during both excitation and inhibition of the adductor and abductor muscles of the claws of intact animals or of animals with one nerve trunk cut and after electric stimulation of the nerve in the ischiopodite. It was found important in the experiments with intact animals to avoid fixation. Von Holst (1934, p. 247) has shown for Carcinus that fixation of any extremity alters the nature of the movements of all the limbs.

Figures 3a–d are electric records taken from the adductor of the claw of the same intact animal to be sure that the several action potentials were comparable.

Record a shows reflex opening; b shows reflex closing; c shows reflex closing, followed by opening; d shows opening on faradic stimulation.

Figure 4 gives the reactions of the closing muscle after one of the nerve trunks has been cut.

4a shows negative action potentials related to single make and break shocks on stimulating the thick trunk. Here it is clearly shown that the latency for excitation varies with the strength of the stimulation,—in some cases from 30 to 90 milliseconds (ms.). For reflex closing of a claw with a single nerve trunk see figure 8.

4b shows reflex inhibition when only the thin nerve trunk remains; a slight positive deflection appears.

4c shows inhibition after electric stimulation. The latency for inhibition varied from 50 to 90 ms. in several experiments.

4d should be compared with figure 7a, which shows the reactions of the opening muscle with its excitatory nerve intact; figure 4d shows reflex inhibition after cutting the thin nerve trunk. In this case the animal showed these reactions during two days after preparation.

The reaction of the opening muscle upon electric stimulation of the thin nerve after section of the thick nerve is shown in figure 7, and discussed on page 230.

Figure 4e is a record of the reaction when all nerves are intact. It shows

a mixture of both excitatory and inhibitory components. The recording electrodes are placed in such a way that an electro-positive state of the muscle gives a deflection upward and an electro-negative state downward. Therefore we can conclude that in contraction the muscle becomes negative and in relaxation positive in respect to the surrounding tissue. In nerve centers an electro-negative state is found during excitation and an electro-positive state during inhibition (Eccles, 1934; Hughes and Gasser, 1934).

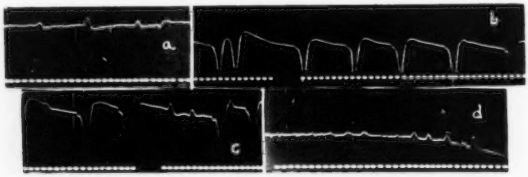

Fig. 3. Cambarus, intact animal. A pair of chlorided silver recording electrodes in adductor. In this and the succeeding records the cathode ray oscillograph is used; 1 mm. = 100μv. Time interval = 5 ms. (Every $\frac{1}{4}$ sec. 4 beats of time signal are omitted.) Faradic stimulation: coil distance = 10 cm.

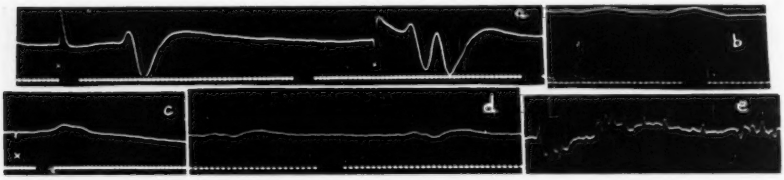

Fig. 4. Homarus. Recording with chlorided Ag-electrodes. Time interval = 5 ms. Ground electrode distal. No antiblock condenser. *a*. Recording electrodes in adductor. Thin nerve trunk cut in pro-carpopodite. Make and break shock [shown by artefacts (crosses) in record]. Coil distance = 9 cm., 1 mm. = 100 μv. *b*. Recording electrodes in adductor. Thick nerve trunk cut. Reflex stimulation; 2.5 mm. = 100 μv. *c*. Recording electrodes in adductor. Thick nerve trunk cut. Electrical stimulation; coil distance = 8 cm. 1 mm. = 100 μv. *d*. Recording electrodes in abductor. Thin nerve trunk cut. Reflex stimulation; 2.5 mm. = 100 μv. *e*. Cambarus, intact animal. Recording electrodes in adductor. Reflex stimulation; 0.5 mm. = 100 μv.

It appears from the above results that the muscles of the crustacean claw show similar electrical states: according to the theory of chemical mediation between nerve and muscle this can be interpreted as signifying that the equilibrium between inhibitory and excitatory processes can be changed in either direction by separate nervous actions.

V. All investigators mention the fact that, after section from the body, a crustacean claw cannot be considered normal for a time varying from 5 to 60 minutes, as it shows various types and degrees of spontaneous activity.

There are at least three possible sources of this spontaneous activity.

a. The cut end of the nerve may set up rhythmic impulses in the muscles (Barnes, 1930) or may change the tonus in some unknown way (von Uexkull and Tirala, 1915).

b. The nerve reticula in the joints may cause, according to Tonner, the changes in the muscle.

c. The muscle itself may be the source of the activity. Its properties might be supposed to change following the cessation of its normal blood supply.

There are several incongruities in the literature, hence it is not yet possible to accept any of the above possibilities as proved. Hoffmann (1914) has already recorded that after cutting the claw the muscle shows several sets of action potentials. Barnes (1930) mentions that he has obtained discharges of nerve impulses lasting from 5 to 20 minutes, accompanied by contraction in the muscles. Sometimes the nerve discharges lasted a few seconds longer than the visible contraction. He gives no figures of these muscle action potentials. In his later papers he gives only one record.

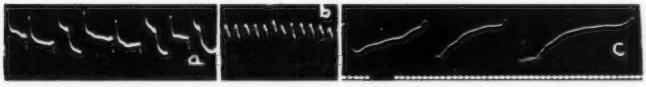

Fig. 5

Fig. 6

Fig. 5. Cambarus. *a* and *b*. Isolated claw. Chlorided silver recording electrodes in abductor; 3.5 mm. = 100 μv. Time interval = 5 ms. *c*. Idem. Intact animal; 1.5 mm. = 100 μv.

Fig. 6. Cambarus. Concentric recording electrodes in isolated adductor; 3 mm. = 100 μv.

Figure 5 shows the development of the rhythm of the muscle action potentials when the claw is removed. For comparison, a reflex rhythm of the opening muscle of the intact animal after strong tapping of the tail is given (c). The rhythm of the large action potentials increases from 20 to 150 per second. There are small excursions partly obscured by the large ones. The large and small components can be seen easily in figure 5a. In b they may still be detected on close examination

Assumption *b*, that the high frequency is due to impulses originating in the joint, can be tested only by isolating the muscle as far as possible by cutting the claw between pro- and carpopod and also dissecting the dactylopod so that all joints are removed and only a muscle in its shell with the nerve-endings is left. This preparation also shows the rhythmical action potentials (fig. 6). From this we may infer that the rhythmic discharges do not come from the joint. Our observations do not permit of a decision as between possibilities *a* and *c*, although I am inclined to favor the former.

Wiersma (1933) gives figures of slower rhythmic muscle action potentials during "contractures" following electric stimulation. He concludes that there are two types of action potentials: one larger, corresponding to an initial fast contraction, and several smaller ones for the following "veratrin-like" contracture. Since he used woollen recording electrodes he probably picked up activity of motor units over a wide area.

From my own experiments I can corroborate the above results without postulating his two types of action potentials (fig. 7a-d). The rhythmic action potentials are at least as large as the initial action potentials. There is also a continuous deflection showing that the large spike is due to a more or less complete summation of single action potentials depending on the frequency of the discharges. (Notice the typical "smooth muscle" action potential, characterized by a small initial excursion followed by a larger one, at the end of c and d.)

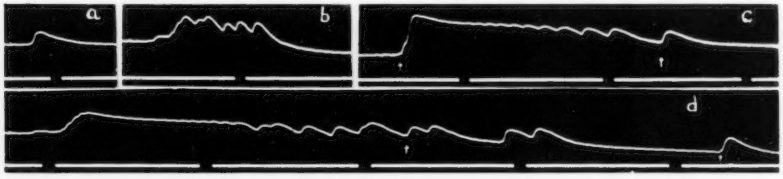

Fig. 7. Homarus. Chlorided silver recording electrodes in abductor. Thick nerve trunk cut. Claw not isolated. Electro-negative stage upward; 1 mm. = 100 μv. Time interval = 5 ms. *a*. Single electric shock. Coil distance = 9 cm. *b*. Idem. Coil distance = 5 cm. *c* and *d*. Idem. Coil distance = 3 cm.

The small components referred to in section III are clearly present in the original record but have been practically obscured by reduction and the limitations of the halftone process of reproduction.

Wiersma's strength duration curves, in addition to his two types of action potentials, provide, according to him, a second argument for an essential difference between a spike and a contracture possibly due to an iterative system. They may be explained as follows: a shock of long duration gives more impulses in the nerve than a briefer shock. The facts that summation can occur at high frequencies (fig. 7) and that separate spikes can be obtained at intervals of 6 to 7 ms., where the mechanical response may be either large or small, shows that the increased responses of longer duration may be due to multiple discharges in the nerve (fig. 8): at least when they last longer than the refractory period of the muscle. A summation of the mechanical effect will be the result. It is not necessary therefore to postulate two types of contraction to account for the phenomena recorded in his curve.

The mechanical records of the muscle activity of the crustacean claw as given in the literature, and also the electrical records as described above, indicate that the crustacean claw may show such phenomena as summation, delay, arrest, alternation, after-discharge, etc. The best description

of several of these phenomena, with convincing mechanical records, can be found in the paper of Knowlton and Campbell (1929). It has also been frequently suggested that iterative systems exist, especially for the slow contractions. Therefore it would be valuable to know whether or not the muscle action potential is related to single or multiple nerve action potentials.

Using the above-described varnish-coated recording needles for single units it was possible to fix them in the claw of the intact lobster on the nerve about 1 cm. from the innervated muscle with only a few sensory nerves in the neighborhood. Both nerve and muscle action potentials are visible when the uncoated area of the needle touches the motor nerve. In other words, the nerve serves as electrode for recording the action potentials of the muscle. It should be noticed that the nerve was not cut and therefore provided a favorable circuit for recording the muscle action potentials. As a control, to show that the muscle action potentials recorded on the nerve correspond to those in the muscle, other chlorided silver electrodes are put directly into the contracting muscle. They are always disconnected from the amplifier when the nerve-muscle action potentials are recorded, to avoid influence on the nerve electrodes.

To apply the recording needles, the joint between pro- and meropod is partly cut. Bleeding is prevented by bringing the animal into such a position that its claw is the highest part of the body. (The animals have low blood pressure.) The preparation remains good for hours and deteriorates only because the needles are fixed in such a way that they lift the nerves a little from the rest of the tissue into the air, to prevent spread of current, and the nerve finally dries. The resulting accessibility to electric disturbances in the air probably causes the 60 cycle artefact which appears in the records. The animal is not seriously injured, for it does not move as violently as it does in response to touching the antennae. Records were taken of spontaneous and reflex movements (touching antennae: opening of claw; touching innerside of claw: closing) and after electric stimulation with small electrodes in the coxopodite.

After an experiment the claw was removed at the joint where autotomy normally occurs to examine the nerve used. Experiments with the closing and opening muscle of the lobster claw are shown in figures 8 and 9. In this both systems show a single muscle action potential after a single nerve action potential. After weak electric stimulation of the nerve with a single shock usually one single discharge is observed in the nerve corresponding with a single muscle action potential. When the strength of the stimulus is stronger other types of response are observed; there may be: *a*, a group of nerve action potentials following directly on the stimulation (fig. 8h), or *b*, several nerve action potentials following in a certain rhythm after stimulation (fig. 8i); *c*, both of these types of response may be combined.

That the disturbance from the same nerve fiber is recorded is probable, for neighboring action potentials are shown as very small spikes.

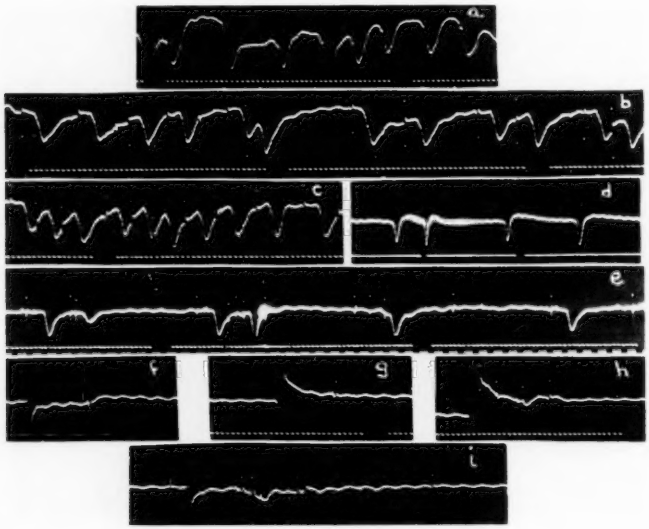

Fig. 8. Homarus. For nerve recording: coated electrodes on thin nerve, ca. 1 cm. from muscle. For muscle recording: chlorided Ag-electodes in abductor. *a–e*: reflex discharges; *f–i*: electric stimulation. The muscle rhythm of *a* corresponds with *b* and *c*; the rhythm of *d*, with *e*. White dots mark the ends of spikes of nerve action potentials; the entire deflections of which are more apparent in the original records. *a* and *d*. Recording electrodes in opening muscle. *b*, *c* and *e*. Recording electrodes on motor nerve of thin system. Mechanical reflex stimulation of high frequency and low intensity in *b* and *c*; and of lower frequency and higher intensity in *e*. *f*, *g*, *h* and *i*. Recording electrodes on motor nerve. Electric stimulation of coxopodite as follows: *f*, coil distance = 12 cm.; *g* = 12 cm.; *h* = 9 cm.; *i* = 6 cm. Distance from stimulating electrodes to nerve recording electrodes about 9 cm.

Fig. 9. Homarus. Chlorided silver recording electrodes in adductor. Coated recording electrodes on thick nerve. *a* and a_1: records of nerve action potentials, followed by muscle action potentials after reflex stimulation. *b* and *c*: electric stimulation; coil distance = 8 cm. (retouched and non-retouched records).

At very high intensities of reflex or electric stimulation the frequency of nerve discharges can be higher than that of the muscle discharges, presum-

ably because of the slower recovery of the muscle. This can explain apparently complex phenomena.

The sequence of the muscle and nerve discharges is not always the same. Where the nerves themselves serve as electrodes for the muscle action potentials, there are several possibilities of recording, for the nerve can pick up the muscle action potentials of the unit innervated by itself, when this unit is the first one which the nerve approaches closely; or the nerve will pick up the action potentials of muscle units which it approaches closely before entering its own unit. In this case the nerve action potentials can be superposed upon the muscle action potentials, for the rhythm of both remains nearly the same.

I have not tried to record the nerve action potentials which belong to the high frequency rhythms of the muscle after cutting of the claw. Prosser (1934) finds for the chela nerve in response to injury a maximal frequency of 208 per second for a duration of 5 to 17 minutes. In the nerves of the walking legs the same sort of high frequency response followed injury stimulation. It is probable that his high frequency discharges in the nerve correspond to the similar discharges in the muscle as shown in figure 5. Because of the difference in recovery times, however, the frequency of nerve impulses may be higher than that of the muscle responses.

It would be interesting to investigate in how far a Wedensky effect is possible in a muscle that can be excited by nerve impulses during its recovery period (fig. 7). So far there is no evidence for this effect in this case. This problem and also the simultaneous activity of both nerve systems can be attacked only with a double set of oscillographs.

SUMMARY

It is shown on several grounds that the muscles of the claw of Crustacea are physiologically intermediate between the mammalian striated and smooth muscles, e.g., by their reaction to adrenalin and acetylcholin and by the double action potentials following a single electrical shock.

Inhibition in the muscle is associated with relative electrical positivity of the muscle, and excitation, with negativity.

The supposition of two contractile systems in the same muscle is not corroborated.

Both the adductor and abductor of the claw can show one single nerve action potential followed by one single muscle action potential, both on reflex stimulation and on application of single electrical shocks.

More complex phenomena can be analyzed into their components, taking in account the fact that the frequency of nerve discharges can be higher than that of the muscle discharges because of the slower recovery of the latter.

An analysis is possible only with the simultaneous recording of both nerve and muscle activity.

I wish to thank Doctor Forbes and Doctor Davis for their hospitality which has enabled me to obtain the foregoing results and for their advice. Also I wish to thank Doctor Rosenblueth and Miss E. Lambert for their help, and especially Doctor Prosser for help in the preparation of this paper.

I am further much indebted to "Curatoren F. C. Dondersfonds" for the stipend that has made it possible for me to carry out the investigations at the Harvard Medical School, Boston.

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EFFECTS OF INGESTION OF NUTRITIVE AND NON-NUTRITIVE LIQUIDS UPON DIURNAL VARIATIONS IN WEIGHT LOSS

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Weight loss changes following the ingestion of large and small meals have been shown by the writer (3) to parallel closely the variations in oxygen consumption which have been found to occur under these circumstances. In order to verify this relationship and to provide additional data on fluctuations in rate of weight loss following the consumption of other types of meals, the present investigation was undertaken. Concentrated food is highly standardized, easily digested and, when dissolved, can be ingested without mastication. In addition, the percentages of protein, carbohydrate, and fat have been previously calculated for this preparation. The effects on weight loss of the concentrated food itself can best be determined when control readings are taken on the variations in rate of weight loss following the drinking of an amount of water equivalent to that in which the concentrated food was dissolved when ingested. The data on the changes in rate of weight loss following water ingestion should be of further significance for comparison with earlier studies concerning the effect of water upon other metabolic indices such as cardiac output and oxygen consumption. That these various indices do not always vary concomitantly is shown by the marked discrepancy between the results of Jarisch and Liljestrand (5) and those of Grollman (1).

The controversy concerning the effects of water ingestion upon insensible weight loss has been traced by Gilman and Barbour (11). Investigators such as Erismann (10) and Moog and Nauck (8) report extensive changes in rate of weight loss, while Schwenkenbecher (9) and Jores (12) find the rate little affected by the administration of large quantities of water. The degree of hydration is suggested as an important variable in the rate of weight loss by Manchester, Husted and McQuarrie (7) and their results were confirmed by Gilman and Barbour (11) in a careful study of blood osmotic pressure changes.

[1] The experiments reported in this paper were performed in the Psychology Laboratory of Northwestern University. The writer wishes to thank Dr. Francis G. Benedict for the loan of the apparatus and Dr. G. L. Freeman for advice and assistance.

METHOD. As in our earlier experiments (3, 4), weight loss determinations were obtained by the use of an extremely sensitive Sauter balance. The cot upon which the subject reclined was suspended directly from one beam of the scales. Counterbalancing weights were placed upon a pan suspended from the opposite beam.

Ten subjects were employed. They were normal, healthy males, ranging in age from 17 to 34 years (mean 24.8 years). Their average height was 68.9 inches (range 65 to 70 inches), and their average weight, 132.8 pounds (range 125 to 146 pounds). The subjects were in a basal, post-absorptive state at the commencement of the tests. No physical exertion was allowed between weighings, but the subjects were permitted to read. The subjects wore light, loose clothing and their shoes were removed. Room temperature was maintained between 70 and 74 degrees Fahrenheit.

An entire day was devoted to accustoming the subjects to the apparatus and routine before the experimentation began. The subjects were divided into two groups. The first group was given concentrated food dissolved in water on the first and third days of the experiment proper and water alone on the second and fourth days. The subjects in the second group were given water on the first and third days and concentrated food and water on the second and fourth.

The concentrated food was prepared and generously supplied by Horlick's Malted Milk Corporation. According to their analysis the product contains:

	per cent
Moisture	3.06
Protein	16.35
Maltose and lactose	49.15
Dextrin	18.80
Fat	8.78
Ash	3.86

On the basis of this analysis the calorific yield will be as follows for 100 grams:

16.35 grams of protein will yield	67.03 calories
8.78 grams of fat will yield	81.65 calories
67.95 grams of carbohydrates will yield	278.58 calories

The experimental meal consisted of 100 grams of the concentrated food dissolved in 230 cc. of water. On the alternate days 230 cc. of water were administered. The liquids were ingested at twelve o'clock noon and weight loss readings were taken hourly thereafter.

EXPERIMENTAL RESULTS. In table 1 are presented the number of grams of weight lost during successive hours following the ingestion of the concentrated food. The amount of change (in grams) from hour to hour is shown. The extent of individual difference can be judged from the ranges

and probable errors. The ratio (in per cent) which each hour's loss bears to the basal, pre-ingestive rate is also given. Each determination in the tables is based upon two days' records of ten subjects.

Table 2 shows the changes which take place in weight loss following the drinking of 230 cc. of water. The data from both table 1 and table 2 are presented graphically in figure 1.

TABLE 1

Weight loss (in grams) at successive hours following the ingestion of 100 grams of concentrated food dissolved in 230 cc. of water

Averages based upon two days' records of ten subjects

	PRE-INGESTION (BASAL)	HOURS AFTER FOOD INGESTION				
		1	2	3	4	5
Grams lost per hour	38 32	42 66	44 67	43 37	41 32	40 33
Range (in grams)	34–45	37–46	38–48	38–48	35–47	31–42
Change (in grams) from preceding hour		+4 34	+2 01	−1 30	−2 05	−0 99
P.E.$_M$ of change		0 53	0 58	0 41	0 50	0 42
Percentage of base rate	100 00	111 32	116 57	113 19	107 83	105 26

TABLE 2

Weight loss (in grams) at successive hours following the ingestion of 230 cc. of water

Averages based upon two days' records of ten subjects

	PRE-INGESTION (BASAL)	HOURS AFTER WATER INGESTION				
		1	2	3	4	5
Grams lost per hour	37 21	39 86	38 51	37 47	37 52	36 96
Range (in grams)	32–43	32–48	34–42	35–41	32–40	33–41
Change (in grams) from preceding hour		+2 65	−1 35	−1 04	+0 05	−0 56
P.E.$_M$ of change		0 56	0 52	0 45	0 39	0 54
Percentage of base rate	100 00	107 11	103 49	100 70	100 84	99 32

The difference between the percentages of increase in the first and second table, which presumably represents the dynamic action of the concentrated food itself, is given in table 3.

Discussion. According to the data presented in table 2 the rate of weight loss is at a maximum during the first hour following the ingestion of the 230 cc. of water. At that time the increase in weight lost over the basal rate amounts to a little over 7 per cent. This is to be compared with the value of 17 per cent which Himwich and Haynes (2) obtained upon oxygen consumption with rats. Their results were obtained, however, when water was injected and the muscular movement of the experi-

mental animals may be expected to account for some of this rise. The work of Grollman (1) indicates that the maximum increase in respiratory metabolism occurs almost immediately after the ingestion of water. He reports an average increase in metabolism of 8 per cent following the drinking of 1000 to 1200 cc. of water. Because the amount of water which he administered in his experiments was greater than in ours, the increase

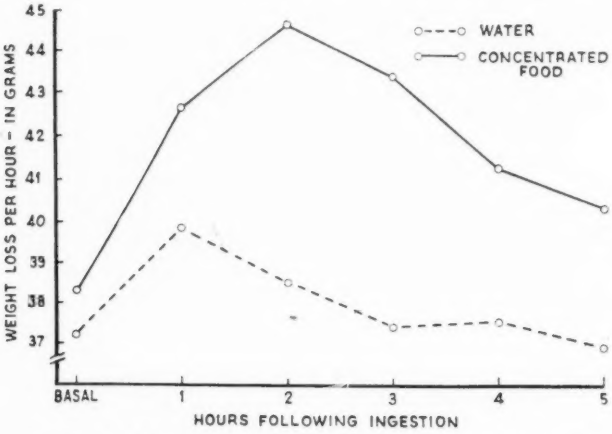

Fig. 1. Showing weight loss (in grams) at successive hours following the ingestion of 100 grams of concentrated food dissolved in 230 cc. water, and of 230 cc. of water alone. "Basal" represents pre-ingestive rate.

TABLE 3

Difference (in per cent) between weight loss changes following ingestion of 100 grams of concentrated food dissolved in 230 cc. of water and those following ingestion of 230 cc. of water alone

Averages based upon two days' records of ten subjects

	PRE-INGESTION (BASAL)	HOURS AFTER INGESTION				
		1	2	3	4	5
Difference (in per cent)	0.00	4.21	13.08	12.49	6.99	5.94

which he reports is actually relatively smaller than the increase which we have found. Our results shed little light upon the perplexing problem of the nature of this increase. Lublin (6) considers the rise in respiratory metabolism following water ingestion as being due to the extra energy required by the kidneys to excrete the water. This factor might also be reflected in the rate of weight loss. Future work devoted to this problem might profitably be directed toward a careful comparison of the effects of injection as compared with ingestion of water upon oxygen consumption and weight loss.

It will be observed that the maximum rate of weight loss after the ingestion of the concentrated food dissolved in water, occurs during the second hour (table 1). At this time the weight loss is about 16½ per cent above the basal rate. During the fifth hour the rate is still about 5 per cent above the basal.

When we deduct the amount of change due to the water from the change due to the concentrated food and water combined, we find the maximum effect on rate of weight loss of the concentrated food itself occurring in the second hour. During the second hour, the difference between the weight loss changes following the ingestion of the concentrated food dissolved in water and those following the ingestion of water alone amounts to 13 per cent.

SUMMARY

Hourly determinations of weight loss were made upon ten male subjects following the ingestion of 100 grams of concentrated food dissolved in 230 cc. of water. A sensitive Sauter balance was employed for weighing. The maximum increase in weight loss was registered during the second hour, when the rate was 16½ per cent above normal. There was a gradual decline in rate of weight loss thereafter, and by the fifth hour the rate was only 5 per cent above basal. Similar determinations of weight loss were made following the intake of 230 cc. of water alone. The rate of loss here increased about 7 per cent during the first hour and decreased slightly thereafter. This change is comparable to the increase of from 5 per cent to 10 per cent in respiratory metabolism following water ingestion reported by Grollman (1). During the second hour following the ingestion of concentrated food and water the rate of weight loss was 13 per cent higher than following the ingestion of water alone. The greater increase in the rate of loss following the ingestion of the concentrated food dissolved in water as compared with that following the ingestion of water alone is presumably due to the dynamic action of the nutritive elements in concentrated food.

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KINETICS OF THE ELIMINATION OF SUBSTANCES INJECTED INTRAVENOUSLY (EXPERIMENTS WITH CREATININE)

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It was shown in a previous publication (Dominguez and Pomerene, 1934) that the rate of excretion of ingested creatinine is proportional to the concentration of ingested creatinine in the plasma, after the equilibrium between the plasma and tissue fluids has been practically attained. From this fact an expression was derived (Dominguez, 1934a) for the volume of body fluids in which creatinine is distributed during equilibrium, assuming the concentration in this volume equal to that of the plasma. This result was extended to include the case of substances in part excreted according to the same law and in part utilized in the body, but the proper method to estimate the utilization constant (Dominguez, 1934b) was not given, pending an investigation into the kinetics of the elimination of substances injected intravenously.

The latter will be presented now, together with an application of it to the elimination of creatinine. Since it has been found that this substance is practically all excreted, the question of partial utilization will be left out of consideration at this time.

THEORETICAL CONSIDERATIONS. It is proposed to determine the equation representing the time change of creatinine concentration in the plasma following the intravenous injection of this substance. In this study it will be assumed that the substance distributes itself so rapidly in the circulatory system that the initial unevenness in concentration may be neglected.

Let us call x the concentration of creatinine in the plasma, in milligrams per 100 cc., and y the rate of excretion of creatinine, in milligrams per minute. From previous work we can write

$$(y - y_e) = A(x - x_e), \tag{1}$$

where y_e and x_e are the rate of excretion and the plasma concentration, respectively, of endogenous creatinine. In order to simplify the sym-

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bolism we may write $y - y_e = \eta$, $x - x_e = \xi$, and equation 1 becomes

$$\eta = A\,\xi. \tag{2}$$

A, the excretion constant of creatinine, represents the rate of excretion per unit plasma concentration. We know also that, at equilibrium between plasma and tissues, the following relation is satisfied (Dominguez, 1934a),

$$-\frac{V}{100}\frac{d\xi}{dt} = \eta\,, \tag{3}$$

or, from equation 2,

$$-\frac{V}{100}\frac{d\xi}{dt} = A\xi\,, \tag{4}$$

where V is the volume of fluids in which creatinine is distributed (volume of distribution).

The solution of equation 4 is

$$\xi = ae^{-\alpha t}, \tag{5}$$

in which

$$\alpha = \frac{6A}{V}\,, \tag{6}$$

when t is determined in hours and V in liters. The constant a is the constant of integration, and it represents the initial concentration of creatinine in the plasma. By initial concentration is meant not the concentration at the beginning of the experiment, but the concentration found—or computed—at the time of equilibrium. In our experiments on a human being (Dominguez and Pomerene, 1934) the time of equilibrium was found to be between 2.5 and 3 hours after the ingestion of the substance.

From equation 6 one can compute the volume of distribution V once α and A have been determined.

When creatinine is injected intravenously the term representing the diffusion of the substance in the tissue juices has to be introduced in equation 4. If we split the volume V in two parts, namely, the equivalent plasma volume P, and the tissue volume T, and call z the concentration in the tissue volume, equation 4 becomes

$$-\frac{P}{100}\frac{d\xi}{dt} = A\xi + \frac{T}{100}\frac{dz}{dt}\,. \tag{7}$$

After a sufficient time has elapsed, z becomes equal to ξ and, therefore, from equation 7,

$$-\left(\frac{P}{100} + \frac{T}{100}\right)\frac{d\xi}{dt} = -\frac{V}{100}\frac{d\xi}{dt} = A\xi\,,$$

as demanded by equation 4.

The solution of equation 7 requires a knowledge of the rate at which the concentration changes in the tissues. It is not unreasonable to suppose that the rate at which the substance diffuses from the plasma into the tissues will be a function of the concentration difference between the two volumes, and since by virtue of such diffusion the concentration difference will become smaller and smaller, we make the fundamental assumption that the rate of diminution of the concentration difference is proportional to the concentration difference itself. In symbols,

$$-\frac{d}{dt}(\xi - z) = \beta(\xi - z)\,. \tag{8}$$

The integral of equation 8 is

$$\xi - z = ce^{-\beta t}, \tag{9}$$

where c is the constant of integration. Since at zero time the concentration in the tissue volume is zero,[3] $c = \xi_o$, that is, c is the concentration in the plasma at zero time.

From equation 9 it follows, by differentiation, that

$$\frac{dz}{dt} = \frac{d\xi}{dt} + c\beta e^{-\beta t}\,. \tag{10}$$

Substituting this value of (dz/dt) in equation 7, we get, after rearranging terms,

$$-\frac{V}{100}\frac{d\xi}{dt} - A\xi = \frac{Tc\beta}{100}e^{-\beta t}\,, \tag{11}$$

the solution of which is of the form

$$\xi = ae^{-\alpha t} + be^{-\beta t}, \tag{12}$$

where α is again given by equation 6 and β by the equation

$$\beta = \frac{6Ab}{Vb - T(a+b)}\,. \tag{13}$$

It will be shown that the experimental data are in satisfactory agreement with equation 12, but before proceeding with the experiments the theoretical part will be developed further in order not to break the continuity of the argument.

[3] In this development z is considered as the concentration of exogenous creatinine in the tissue volume, just as ξ represents the concentration of exogenous creatinine in the plasma.

Substituting the expression for ξ from equation 12 in equation 9, we get

$$z = ae^{-\alpha t} + (b - c)e^{-\beta t}, \tag{14}$$

but at zero time $z = 0$, therefore, $a + b - c = 0$ and equation 14 becomes

$$z = a(e^{-\alpha t} - e^{-\beta t}). \tag{15}$$

By comparing equation 12 and 15, we see that whereas the plasma concentration ξ decreases steadily from the beginning to the end, the concentration in the tissues z increases at first, reaches a maximum and then decreases, becoming asymptotically equal to that of the plasma. The concentration in the tissues is consequently greatest some time before the equilibrium between the plasma and the tissues may be said to have been established.

That the time of maximum concentration is independent of the quantity of creatinine injected is shown by differentiating equation 15 with respect to time, equating the derivative to zero and solving for t. The result is

$$t_m = \frac{\log \beta - \log \alpha}{(\beta - \alpha) \log e}, \tag{16}$$

which depends only on the essential constants α and β, and is therefore independent of the quantity injected.

Since A has been assumed constant during the whole experiment, it is obvious from equations 2 and 12 that the time change of the rate of excretion will be of the form

$$\eta = A(ae^{-\alpha t} + be^{-\beta t}). \tag{17}$$

This result has also been tested by experiment as will be shown in the sequence. From equation 17 it appears that, if creatinine is not destroyed in the body in appreciable quantity, the integral of η from zero to infinity should be equal to the quantity injected. If we designate this quantity by G, we should have

$$\int_0^\infty \eta \cdot dt = 60\, A \left(\frac{a}{\alpha} + \frac{b}{\beta}\right) = G \tag{18}$$

if α and β are computed in $(\text{hours})^{-1}$.

Any objection that could be raised against the use of an infinite time in a biological experiment is answered by the consideration that in a relatively short time—in our experiments, less than 48 hours—the integrand in equation 18 becomes unmeasurably small and the tail end of the integral can be neglected.

When the substance to be studied is quantitatively excreted by the kidney, equation 18 affords a convenient check on the computation of the constants, a check which does not exist for the plasma data.

After A and the constants of equation 12 have been computed, the equivalent plasma volume P and the tissue volume T can be calculated from equations 6 and 13. The solutions are

$$P = \frac{6\,A\,(a\beta + b\alpha)}{\alpha\beta\,(a + b)}, \tag{19}$$

$$T = \frac{6\,Ab\,(\beta - \alpha)}{\alpha\beta\,(a + b)}. \tag{20}$$

It should be noted that the expressions for P and T are given not only in terms of A, α and β, but also of a and b. The last two constants depend on the quantity of substance injected, as can be seen in equation 18, while the first three do not. Since P and T cannot depend on this quantity, it is necessary that (b/a) be independent of the quantity of substance injected. This condition, which is also sufficient, has been verified for creatinine. Calling k the ratio (b/a) and introducing it in equations 19 and 20, we obtain finally

$$P = \frac{6\,A(\beta + k\alpha)}{\alpha\beta\,(1 + k)}, \tag{21}$$

$$T = \frac{6\,Ak\,(\beta - \alpha)}{\alpha\beta\,(1 + k)}, \tag{22}$$

where the two volumes are now expressed in terms independent of the quantity injected.

The sum of P and T can be seen, with the help of equation 6, to be equal to V, as it should by definition.

In practice it will be found more convenient to calculate these volumes from the equivalent relations

$$V = P + T = \frac{6\,A}{\alpha}, \tag{23}$$

$$\frac{T}{P} = \frac{k\,(\beta - \alpha)}{\beta + k\alpha}. \tag{24}$$

The ratio of the two volumes, being independent of A, could be calculated from observations on the plasma alone, but for the absolute value of the volumes it is necessary to make observations on the rate of excretion also.

Experimental part. The experiments were carried out on three dogs. The animals had all been on a standard diet (Purina Dog Chow) for periods of several months to a year prior to the experiments and were kept on this diet between experiments. Food was withdrawn the night before and during each experiment, but during some of the experiments water (200 to

400 cc.) was given by stomach tube at intervals of about 2 hours in order to increase the urine flow (see table 1).

The experiments are very simple. At a given time in the morning the bladder of the dog is catheterized and a known quantity of creatinine is injected quickly into the jugular vein. At known intervals thereafter samples of blood are withdrawn from the vein and the urine is collected by catheter. In the beginning the samples are taken at relatively short intervals, 15 minutes or so; later, when the concentration in the plasma changes less rapidly, every hour. The experiments last from eight to ten hours, but in most instances the urine was collected at the end of 24 hours,

TABLE 1

Data of experiment 3, dog 2, February 12, 1935

EXPERIMENTAL DETAILS	INTERVAL OF URINE COLLECTION	URINE VOLUME	URINE CREATININE	TIME OF BLOOD COLLECTION	PLASMA CREATININE
		cc	*mgm. per 100 cc.*		*mgm. per 100 cc.*
Intravenous injection of 76.4 cc. of 8.72 per cent solution of creatinine (Eastman Kodak Co.) in distilled water, from 8:52½ to 8:54 a.m. At 9:56 a.m. and at 1:10 p.m. 400 cc. of water were given by stomach tube. At 8:54 a.m., Feb. 13, 1935, 205 cc. of urine, containing 619 mgm. of creatinine per 100 cc., were obtained by catheter. No urine was voided during the night	8:52– 9:09	53 0	1564	8:51	1 00
	9:09– 9:24	18 0	2734	9:04½	82 3
	9:24– 9:39	12 2	2993	9:10	71 3
	9:39– 9:54	8 9	3314	9:26½	43 3
	9:54–10:24	23 0	2485	9:39½	42 2
	10:24–10:54	84 0	441	9:54	32 7
	10:54–11:54	122 0	581	10:25	25 3
	11:54–12:54	28 0	1788	10:55	21 6
	12:54– 1:54	58 0	766	11:55	18 0
	1:54– 2:54	140 0*	157	12:54½	13 6
		33 0	396	1:54½	9 70
	2:54– 3:54	31 0	1013	2:56	8 80
	3:54– 4:54	20 5	1147	3:55	7 67
				4:57	6 09

* Volume of urine collected from the cage. All the others by catheter.

and in a few instances until 48 hours from the time of the injection. Between experiments a sufficient number of observations was made on the rate of excretion of endogenous creatinine and also on the concentration of endogenous creatinine in the plasma, in order to estimate the mean value of each. These mean values are treated as constants.

In all of these experiments the concentration of creatinine in plasma and urine was determined after the samples had been renumbered by an independent observer. The chemical methods employed have been published previously (Dominguez and Pomerene, 1934).

An illustrative protocol is given in table 1.

The mean rate of excretion, y_e, and the mean plasma concentration, x_e,

of endogenous creatinine are given in table 2, together with the number of observations and the standard error of the mean. The large standard error in the output of dog 3 is due to a single large value of 0.80 mgm. per minute. From subsequent experience with this dog, as well as from the

TABLE 2

Data on endogenous creatinine (mean values)

DOG NUMBER	CONCENTRATION IN PLASMA, x_e	NUMBER OF OBSERVATIONS	STANDARD ERROR OF MEAN	RATE OF EXCRETION, y_e	NUMBER OF OBSERVATIONS	STANDARD ERROR OF MEAN
	mgm. per 100 cc.			*mgm. per min.*		
1	1.08	11	0.0408	0.506	9	0.0228
2	1.10	9	0.0210	0.478	9	0.0173
3	0.92	8	0.0275	0.427	8	0.0597

TABLE 3

Time change of creatinine concentration in the plasma, ξ, after intravenous injection

$$\xi = ae^{-\alpha t} + be^{-\beta t}$$

EXPERIMENT NUMBER	DOG NUMBER	DATE	BODY WEIGHT	CREATININE INJECTED	A	a	α	b	β
			kgm.	*grams*		*mgm. per 100 cc.*	*(hour)*$^{-1}$	*mgm. per 100 cc.*	*(hour)*$^{-1}$
1	1	7- 6-34	20.8	7.05	0.642	28.52	0.223	83.28	1.80
2	2	1-23-35	20.2	4.849	0.451	26.77	0.205	60.52	2.76
3	2	2-12-35	20.2	6.66	0.592	33.00	0.234	74.00	2.46
4	2	3-29-35	20.2	2.02		12.05	0.189	26.32	2.99
5	3	3- 1-35	18.6	6.138	0.589	34.65	0.246	77.34	2.78
6	3	3-26-35	18.4	1.737	0.826	8.57	0.355	20.09	2.47
7	3	4- 2-35	17.8	1.78	0.706	9.05	0.301	21.28	2.30

A from the equation $\eta = A\xi$, η being the rate of excretion of exogenous creatinine, in milligrams per minute, and ξ the concentration of exogenous creatinine in the plasma, in milligrams per 100 cc.

The constants a, α, b and β were calculated from the ξ and η data in experiments 1, 2, 3, 5 and 6, from the ξ data alone in experiment 4, and from the η data alone in experiment 7.

The constant A cannot be calculated in experiment 4 because in this experiment no observations were made on the rate of excretion η. See text for an approximate way to estimate A in this experiment.

data of other dogs of comparable size, this value is probably much too high. However, its rejection produces only a small change in the mean.

The weight of the dogs and the quantity of creatinine injected are shown in table 3. The duration of the injection varied between one half and one and one-half minutes, but for the purpose of computation the experiment was assumed to begin with the beginning of the injection.

Computations. The first step in the computations was to determine the ratio (η/ξ), by linear interpolation either at the middle of the intervals of urine collection or at the times of the blood samples, and to examine whether the ratios showed a systematic deviation from their mean value in the course of the experiment.

In the work we are reporting here the mean value of the ratios could be assumed constant during each experiment, although it varied in the different dogs and in the different experiments (see the values of A in table 3). The η-points of one experiment were then multiplied by the reciprocal of the corresponding A, and the two sets of points, ξ and η/A, were then handled as one set for the determination of the constants in equation 12. This procedure was adopted in five out of seven experiments after the calculation of the constants from the ξ and η points, separately, showed that the two values of α in a pair of corresponding curves (and similarly for β) were sufficiently close for the purpose of this investigation. In experiment 4 (table 3) only the plasma curve was calculated, because the dog developed frequent micturition of a blood-stained urine and on this account no observations were made on the rate of excretion of creatinine. Finally, in experiment 7 (table 3) only the η curve was calculated, because the excretion data are much more smooth than the plasma concentrations, and because the ξ residuals from the curve $\eta/0.706$ are so nicely distributed that no further calculation was deemed necessary.

The function to be tested is the sum of two exponentials (equation 12). The exponent β has been found much larger than α, so that after a relatively short time the term $(be^{-\beta t})$ becomes practically negligible. From this time on the changes proceed as a single exponential.

The general course followed was to plot the logarithms of one of the quantities studied (minus its mean endogenous value), with the time as axis of abscissae, and to calculate a and α approximately, either by selected points or by the method of averages, from the linear portion of the graph. The value of $ae^{-\alpha t}$ was then computed at the corresponding times and the differences $(\xi - ae^{-\alpha t})$ were calculated. These differences represent points on the simple exponential $be^{-\beta t}$, as can be seen from equation 12, and the process used to determine a and α was now repeated for b and β. With the help of these constants, the residuals were calculated and the curves plotted. The calculations are the same whether we take the ξ and η points separately or jointly.

The curves cannot be synchronized to yield a mean curve. The computed values of the constants therefore will be considered only as rough approximations useful nevertheless in helping to establish relations between quantities. As to the mean values of the quantities themselves, the results of the calculations may be expected to give an idea of their order of magnitude.

The numerical results are presented in table 3. The arbitrary constants a and b correspond to the plasma curves (equation 12), even in the experiment in which only the η curve was calculated (expt. 7). In this case the constants a and b were computed from the relation $\xi = (\eta/0.706)$ in order to facilitate comparison. It will be seen that a and b depend on the quantity of creatinine injected, whereas α and β do not. Likewise A is independent of the quantity injected.

Other results will be examined in more detail in the following paragraphs.

Quantitative excretion of creatinine. The integration of the η curve (equation 18) between zero time and infinity assigns a theoretical upper limit to the total excretion of the substance. From the data in table 4 one can see that the amount excreted is, on an average, 92.3 per cent of the amount injected.

TABLE 4

Total excretion of exogenous creatinine after intravenous injection

EXPERIMENT NUMBER	DURATION OF EXPERIMENT, t	AMOUNT EXCRETED IN TIME t			$\int_0^\infty \eta\, dt$	
		Found	Calculated			
	minutes	*grams*	*grams*	*difference in per cent of quant. found*	*grams*	*per cent of quant. injected**
1	1440	6.654	6.681	+0.40	6.704	95.1
2	1441	4.130	4.093	−0.90	4.119	84.9
3	1442	6.056	6.060	+0.07	6.078	91.3
5	1441	5.994	5.943	−0.85	5.957	97.1
6	1441	1.553	1.600	+3.03	1.600	92.1
7	1380	1.707	1.665	−2.46	1.666	93.6

* See table 3 for quantity injected.

The amount of creatinine removed with the blood samples accounts for a negligible portion of the loss. Thus, in the first experiment on dog 3, the total amount removed in 13 samples of about 10 cc. each represents only 39.0 mgm. of exogenous creatinine, that is 0.6 per cent of the quantity injected. Since the difference is systematically by defect, it might be asked whether we have introduced systematic errors in the fitting. The integrals calculated to the end of the total time of collection of urine (between 1380 and 1442 minutes, see table 4) show that the mean per cent difference is -0.15, which is not significantly different from zero. The average difference is ± 1.3 per cent.

Since the urine is collected by catheter, and a small amount of urine adheres to its wall, we have made a few determinations on the washings of the catheter, with the results shown in table 5. The loss of creatinine in the catheter averages 1.3 per cent of the amount excreted, it being somewhat higher with a low diuresis than with large urine flow, as was to be expected.

In other words, the theoretical amount excreted (calculated from the curves) is, on an average, 94.2 per cent of the quantity injected, if we subtract from the latter the small amount removed with the blood samples and that remaining in the catheter. At this point it is well to call attention to the fact that the calculated excretion up to the end of 24 hours involves considerable *extrapolation*, because the constants have been computed only from the data corresponding to the first eight hours following injection.

Since creatinine may be lost in the urine on standing, especially in alkaline urines, and since the substance may be excreted in minute amounts by other channels, it would seem at the present time unnecessary to discuss the question of the transformation of creatinine in the body. For practical purposes, therefore, we shall set the constant of utilization of creatinine equal to zero, and consequently, the volume of distribution V is actually given by equation 23.

TABLE 5

Quantities of creatinine obtained from washings of the catheter

CREATININE INJECTED	EXCRETION INTERVAL	AVERAGE DIURESIS IN INTERVAL	CREATININE EXCRETED IN INTERVAL	CREATININE IN WASHINGS	PER CENT IN WASHINGS
grams	*min.*	*cc./min.*	*mgm.*	*mgm.*	
1.78	120*	2.35	1022.10	5.25	0.5
5.55	120*	2.29	3884.50	39.7	1.0
1.74	121*	0.96	1067.39	19.6	1.8
4.85	300†	0.67	1605.36	29.88	1.9

* First six catheterizations.

† From the 6th to the 10th catheterization inclusive.

The total amount excreted in these experiments with intravenous injection is somewhat larger than the amount usually excreted after ingestion (see review by Hunter, 1922). The difference is probably to be accounted for either by incomplete absorption or by partial destruction in the bowel.

Essential constants α, β, and k. The constant α represents, in these experiments, the velocity constant of the elimination of creatinine by the activity of the kidney, after the equilibrium between the plasma and the tissues has practically been attained.

The constant β, on the other hand, represents the velocity constant of the equilibration between plasma and tissues. Equilibrium cannot theoretically be established, because no sooner is it reached than it is destroyed by the continued action of the kidney. However, in practice, equilibrium is effected when the difference between the concentration of the plasma and that of the tissues becomes as small as the measure of precision of the observations.

The ratio $k = (b/a)$ has, empirically, the values presented in table 6. Their mean is 2.362, with standard error of the mean = 0.0958. This

result has been obtained from seven experiments in three dogs of comparable size, with a fourfold variation in the quantity of creatinine injected and an almost twofold variation in renal activity (see values of A in table 3).

The tissue concentration. The concentration in the tissue volume T is given by equation 15. In figure 1 the curve representing the changes (calculated) in the tissue concentration is illustrated together with the curves of plasma concentration and rate of excretion.

It was shown in the theoretical part that the maximum concentration takes place before the concentrations in the two volumes become practically equal, and that the time of the maximum is independent of the quantity injected. Substituting the values of α and β from table 3 in equation 16,

TABLE 6

Volume of distribution of creatinine and other quantities derived from table 3

EXPERIMENT NUMBER	V		P		T		k	t_m
	kgm.	*per cent*	*kgm.*	*per cent*	*kgm.*	*per cent*		*hours*
1	17.27	83.0	6.00	28.8	11.27	54.2	2.92	1.32
2	13.20	65.3	4.73	23.4	8.47	41.9	2.26	1.02
3	15.18	75.1	5.67	28.1	9.51	47.1	2.24	1.06
4							2.18	.99
5	14.365	77.2	5.33	28.6	9.04	48.6	2.23	.96
6	13.96	75.9	5.58	30.3	8.38	45.5	2.34	.92
7	14.07	79.0	5.49	30.8	8.58	48.2	2.35	1.02

V = volume of distribution of creatinine.

P = equivalent plasma volume.

T = tissue volume.

The weights of these volumes are expressed in kilograms of water and in per cent of the body weight. In experiment 4 the values of P and T cannot be calculated because no observations were made on the excretion of creatinine.

$k = b/a$, see table 3.

t_m = time of maximum concentration in the tissue volume T, equation 16.

we obtain for the time t_m, the values shown in table 6, with mean = 1.04 hours, and standard error of the mean = 0.0495.

It will be understood that the theory does not postulate a uniform concentration of creatinine in the tissue volume. Should the concentration be found different in various parts of the body, by suitable experiments, the results given here apply nevertheless to the *mean* concentration.

Volume of distribution, V, and its two components, P and T. In the following the volumes will be assumed to be volumes of water, and will be expressed indifferently in liters or in kilograms.

With the help of the constants of table 3 and equation 6, we obtain for V the values presented in table 6. The mean volume V, in per cent of the body weight, is 75.92, with standard error of the mean = 2.41.

The calculated volumes P and T, from equations 23 and 24, are also given in table 6. The mean per cent value of P is 28.3, with standard error = 1.07, and that of T 47.6, with standard error = 1.65.

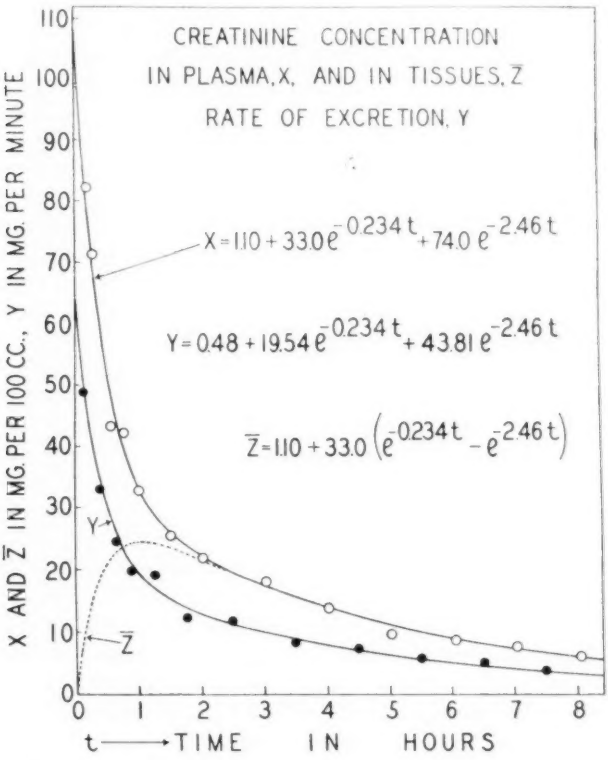

Fig. 1. Time change of the concentration of creatinine in the plasma and of the rate of excretion in the urine, following the intravenous injection of 6.66 grams of creatinine at the time $t = 0$ in dog 2, experiment 3, February 12, 1935. The open circles represent the plasma concentration and the solid circles the average output of creatinine experimentally found (see table 1). The interrupted curve corresponds to the calculated concentration in the tissue volume. This curve has been drawn on an axis 1.10 mgm. per 100 cc. above the x-axis, in order that equation 9 of the text be satisfied. The relation between the z of the text (concentration of exogenous creatinine in the tissue volume) and the $\bar{z}$ of the figure is $\bar{z} = z + 1.10$.

The value of V for experiment 4 cannot be calculated because, for reasons already mentioned, no observations were made on the rate of creatinine excretion. If we assume a total excretion equal to the mean per cent excretion of the other experiments (92.3 per cent of the quantity

injected), we can estimate A by the equation

$$\int \eta \cdot dt = A \int \xi \cdot dt = 60 \times A \left(\frac{12.05}{0.189} + \frac{26.32}{2.99} \right) = 0.923 \times 2.02 \times 10^3 \text{ mgm.}$$

from which we get $A = 0.428$, $V = 67.26$, $P = 24.04$ and $T = 43.22$, the volumes being in per cent of the body weight as before. The values so obtained are very close to the corresponding values in another experiment on the same dog (expt. 2).

The volume P is, therefore, much larger than the plasma volume. For this reason and also because it behaves like a volume of plasma in the theory outlined before, we have called it *equivalent plasma volume*. Just what this volume represents anatomically is a matter of conjecture. Judging from the extreme rapidity with which it enters into equilibrium with the true plasma, as compared with the tissue volume, it would not be unreasonable to suppose that it is made up of the perivascular fluids and lymph together with the plasma itself. In the neighborhood of the vessels, the looseness of the connective tissue, the thinness of the capillary walls, the movements of the vessels, and the formation and propagation of the lymph, will be expected to aid very materially the dissemination of the creatinine molecules. In regions farther removed from the vessels, in the parenchyma of the organs as distinct from the stroma, where the movement of fluids and their solutes is more sluggish, and where the cell boundaries and other barriers may actually impede the motion of the molecules, the diffusion of creatinine probably proceeds more slowly. Whether this anatomical picture corresponds to or underlies the kinetical scheme of elimination developed in this paper, we do not know.

That the volume of distribution of creatinine is greater than the volume of water in the body is certain. In the dog, the quantity of water is about 69.3 per cent of the body weight (Moulton, 1923, data of Pfeiffer) when reckoned on a fat free basis, and since our dogs are not emaciated, a figure of 76 per cent is certainly too high. This would indicate that creatinine penetrates into the non-aqueous portion of the cells, or else it is adsorbed or combined there. The last two possibilities must constitute at the best a loose sort of bond, because creatinine is given off readily up to 93 per cent of the quantity injected.

While the equivalent plasma volume is approximately a volume of water, the tissue volume may not be, if the substance under investigation penetrates into the cells. In other words, different substances penetrating with different facility into the tissues, may nevertheless have identical distributions in the equivalent plasma volume. Thus, the volume of "available water" for the solution of sodium thiocyanate is, from the figures of Crandall and Anderson (1934), of the same order of magnitude as the equivalent plasma volume of creatinine, from which we would infer

that thiocyanate does not penetrate into the tissue volume. A bit of corroborative evidence of this is found in the observation of Folin and Denis (1912), that creatinine goes readily into the muscles (and may even accumulate there, when excretion by the kidneys is prevented), and in the work of Corper (1915), according to which thiocyanate is not found in muscle after intravenous injection.

The equivalent plasma volume may therefore have a real physiological significance.

SUMMARY AND CONCLUSIONS

When creatinine is introduced rapidly into the blood stream it is dispersed very quickly in a large portion of the volume of fluids in which it will be eventually distributed. This portion has been called *equivalent plasma volume*, because it behaves kinetically like plasma. The other portion has been called *tissue volume*. In the early part of the experiment the concentration of creatinine in the plasma diminishes rapidly, partly by the excretory action of the kidneys, partly by the reversible diffusion into the tissue volume. After the (dynamic) equilibrium between plasma and tissues is practically reached, the concentration in the plasma diminishes solely by the action of the kidneys.

The theory is given a mathematical form with the help of 1, the empirical fact of the proportionality between the rate of excretion and the concentration in the plasma, and 2, the assumption that the concentration difference between plasma and tissue volume diminishes at a rate proportional to the instantaneous concentration difference.

An expression is derived for the relation between the plasma concentration of creatinine and the time following intravenous injection of the substance, from which one can determine not only the curve of the rate of excretion by the kidneys, but also that of the concentration of creatinine in the tissue volume. Observations made in the dog both on the plasma concentration and on the output of creatinine are in satisfactory agreement with the theory.

Expressions are also given for the equivalent plasma volume and the tissue volume in terms of the essential constants of the equations for the plasma concentration and the rate of excretion. The equivalent plasma volume, considered as water, is in our experiments 28 per cent of the body weight. It is probably made up of the plasma, the lymph and part of the perivascular fluids. The volume of distribution of creatinine (equal to the sum of the other two volumes) is equivalent to 76 per cent of the body weight, and may be larger than the quantity of water in the body.

The amount of creatinine excreted, calculated from the equation of the rate of excretion, is, on an average, 94 per cent of the quantity injected. It is assumed that this systematic difference is the result of undetected

constant errors, rather than the consequence of a transformation of creatinine in the body.

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